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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, C07H 21/04, C12P 19/34 — A2

(11) International Publication Number: WO 96/15262

(43) International Publication Date: 23 May 1996 (23.05.96)

(21) International Application Number: PCT/GB95/02644

(22) International Filing Date: 10 November 1995 (10.11.95)

(30) Priority Data:
9422814.5 11 November 1994 (11.11.94) GB

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(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD FOR THE DETECTION OF RAS ONCOGENES, IN PARTICULAR THE K-RAS ANCOGENE

(57) Abstract

The invention relates to an oligonucleotide primer sequence for use in *in vitro* amplification, characterised in that said primer sequence is capable of creating a *BstX* I restriction site overlapping codon (12) and/or an *Xcm* I restriction site overlapping codon (13) or a *Bce* 83I restriction site overlapping codon (61) of the wild-type K-*ras* oncogene, methods of using said primer sequences for detecting activating mutations in codons (12 and/or 13 and/or 61) of the K-*ras* oncogene and kits for performing the methods.

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METHOD FOR THE DETECTION OF RAS ONCOGENES, IN PARTICULAR THE K-RAS ONCOGENE

The present invention relates to the detection of mutations in Ras oncogenes, and in particular in the K-ras gene.

Ras oncogenes are implicated in the development of a range of cancers. In particular somatically induced, activating mutations at defined positions in ras genes are believed to be important causative events in the process of tumorigenesis. Ras gene mutations occur in approximately 30% of human tumours, including cancer of the lung, thyroid, colon, rectum, pancreas and breast, and certain melanomas and leukaemias, although their incidence does vary according to tumour type. In addition, certain experimental tumour systems have been shown to be associated with activated ras genes. More specifically, mutations of the K-ras gene have been reported to be as high as 90% in carcinomas of the pancreas, 50% in adenocarcinomas of the lung and 40% in adenocarcinomas of the colon.

Activation of the ras oncogenes appears to be most frequently associated with mutations at position 12, although activation at other positions e.g. positions 13 and 61 is also commonly observed. Thus, in recent years a number of tests for the detection of ras mutations have been developed as a means towards clarifying their functional role in tumorigenic pathways, and indeed for their potential utility in the diagnosis and prognosis of cancer.

Primer-mediated restriction fragment length polymorphism (RFLP) analysis was developed as a rapid, non-radioactive method for fast and simple large-scale detection of mutant ras genes (Kahn et al., 1990, Amplifications, 4, 22-26). This method relies upon the polymerase chain reaction (PCR) for amplification of the ras sequences and upon the introduction of restriction

endonuclease sites permitting selective restriction cleavage of normal (wild type) ras sequences only, and not of mutant ras sequences. The ras gene primers used for PCR amplification in this process incorporate strategic nucleotide substitutions which serve to create restriction sites overlapping potential activating positions, e.g. positions 12, 13 or 61. The diagnostic restriction site of the target codon is lost in the presence of an activating mutation, thereby permitting detection of the mutant sequences.

However, the detection of ras mutations by this procedure is limited because both mutant and normal sequences are amplified by the PCR step, thereby compromising sensitivity (the number of mutant ras sequences, amongst normal, wild type, DNA may be very low). In more recent years therefore, modifications of the RFLP technique have been developed, whereby mutant ras sequences are selectively amplified (see for example Kahn et al., 1991, Oncogene, 6 1079-1083). This greatly enhances the diagnostic capability of the technique and enables the detection of mutant ras alleles in the presence of up to 10^4 wild type sequences.

Nonetheless, although the combination of primer-mediated RFLP analysis with selective amplification of ras mutant sequences permitted a significant advance in ras mutation detection, there is still room for improvement, for example in the sensitivity and specificity of the method, and such improvements are continually being sought. There is therefore a continuing need for improved methods of detecting mutations in ras genes and the present invention is directed towards providing such an improved method.

In particular, we have directed our efforts towards the K-ras gene, and more specifically to the detection of activating mutations at codons 12, 13 and 61 of the K-ras gene, which are believed to be implicated in the development of many common cancers, most notably colo-

rectal cancer. Thus, a novel and advantageous primer system has been designed, for use in amplification or RFLP-based ras mutation detection methods, which permit the detection of mutations at codons 12 and/or 13 of the K-ras gene. A further primer system permits detection of mutations at codon 61.

In one aspect, therefore, the present invention provides an oligonucleotide primer sequence for use in in vitro amplification, characterised in that said primer sequence is capable of creating a BstX I restriction site overlapping codon 12 and/or an Xcm I restriction site overlapping codon 13 of the wild-type K-ras oncogene.

The sequence of the wild type human K-ras gene is shown in Figure 1.

Mediation of the formation of restriction sites by the primers of the invention is achieved by the provision of a mismatch in the primer at a site, which together with bases present in the wild-type gene sequence, creates the desired restriction site.

A primer sequence according to the invention may thus contain a CCA substitution at the final base of codon 8 and the first two bases of codon 9 of the K-ras gene.

When such a primer is used to direct the in vitro amplification (e.g. by PCR) of a wild-type K-ras sequence, two restriction sites are created, one overlapping the first two, potentially activating, bases of codon 12 (and therefore specific for wild-type codon 12) and the other overlapping the first two, potentially activating bases of codon 13, (and therefore specific for wild-type codon 13). Mutations in the final base positions of codons 12 and 13 do not give rise to changes in the amino acid encoded, and hence are not seen as activating mutations.

When, however, such a primer is used to amplify K-ras sequences mutant at activating positions in codons

12 and 13, a restriction site is not created, since the "correct" bases required to complete the restriction endonuclease recognition site are not present. This therefore enables K-ras sequences containing activating mutations at codons 12 and 13 to be distinguished from wild-type sequences, or more importantly, for the wild-type sequences to be removed, thereby enabling selective amplification of the mutant sequences.

Primers according to the present invention are advantageous in that since restriction sites are created overlapping both codons 12 and 13, mutations at one or both of these sites may readily be detected in the same system. Thus, a single primer may be used to detect the presence or absence of mutations at two sites, thereby considerably simplifying and speeding up the procedure. The utilisation of restriction sites at both loci increases the efficiency of screening and indeed for cancers, e.g. colo-rectal cancer, associated with activations in both codons, the accuracy of the technique.

Different cancers may be associated with different mutations in the K-ras gene, which differ not only in the position of the affected codon, but also in their nature, ie. the actual base substitutions involved. Different combinations of mutations may thus be characteristic of different tumours and identifying mutations at all possible positions could aid in the diagnosis of individual tumours. As mentioned above, codon 61 of the K-ras gene is also commonly affected, and is often associated with different cancers to those caused by mutations in codons 12 and/or 13. It would be helpful therefore to be able to identify mutations at codon 61, in addition to those at codons 12 and 13. Advantageously, therefore, the use of the codon 12/13 primer of the invention is combined with the use of a primer designed to detect mutations at codon 61, ie, by the introduction of restriction sites overlapping codon

61 of the wild type K-*ras* gene. Thus for example, a primer may be used which creates a *Bce* 83I restriction site overlapping codon 61.

In a further aspect the invention therefore provides an oligonucleotide primer sequence for use in *in vitro* amplification, characterised in that said primer sequence is capable of creating a *Bce* 83I restriction site overlapping codon 61 of the wild-type K-*ras* oncogene.

Such a primer is advantageous since it covers all possible mutations in codon 61 and thus may be relied upon to give an accurate diagnosis. A representative primer for codon 61 may comprise a sequence corresponding to a C substitution at the second base of codon 60 of the K-*ras* gene.

Identifying different mutations at different positions may be therapeutically very advantageous since it may permit the targetting of the therapy to the particular cancer concerned. Thus for example, therapies may be directed against the particular mutation, eg. by immunotherapy which stimulates T-cells to kill cells carrying the identified K-*ras* mutation. Such immunotherapeutic techniques are described in WO92/14756 of Norsk Hydro AS.

Moreover, there is a value in determining not only the codon in which the mutation has occured but also the actual mutation concerned. As mentioned above certain cancers are associated with particular substitutions and furthermore, for some cancers, such as pancreatic cancer, certain mutations give a better prognosis than others. In other words, the prognosis of a cancer may depend on the precise nature of the mutation. Consequently, determination of the sequence following identification of the affected codon is therefore also of clinical value.

In addition to the immunotherapy mentioned above, such targetted therapies, which the present invention

may enable to be selected, include the use of ribozymes (JP-A-4235919) and anti-sense RNA (WO92/15680). The product of the ras oncogenes is activated by modification, more specifically by farnesylation, before it exerts its effects. An alternative therapy may therefore involve inhibition of farnesyltransferase enzymes (see for example EP-A-537008).

The length of the primers of the invention is not critical as long as it is sufficient for correct annealing to the template to take place. A length of 20 to 35 bases is suitable for example, and a preferred primer may be e.g. 22 to 30 nucleotides long; and may anneal up to, or just before the potential-activating position. For example for positions 12 and 13 such a primer may comprise the 3' terminal sequence: 5'...CCATGGAGCT 3', where bases CCA correspond to the final base of codon 8, and the first two bases of codon 9 of the K-ras gene.

The primer may be provided with means for immobilisation to a solid support to facilitate subsequent handling and manipulation, for example in the amplification and/or detection steps.

The nature of the means for immobilisation and of the support is a matter of choice. Numerous suitable supports, and methods of attaching nucleotides to them, are well known in the art and widely described in the literature. Thus for example, supports in the form of microtitre wells, tubes, dipsticks, particles, fibres or capillaries may be used, made for example of agarose, cellulose, alginate, teflon, latex or polystyrene. Conveniently, the support may comprise magnetic particles, which permits the ready separation of immobilised material by magnetic aggregation.

The solid support may carry functional groups such as hydroxyl, carboxyl, aldehyde or amino groups for the attachment of nucleotides. These may in general be provided by treating the support to provide a surface

coating of a polymer carrying one of such functional groups, eg. polyurethane together with a polyglycol to provide hydroxyl groups, or a cellulose derivative to provide hydroxyl groups, a polymer or copolymer of acrylic acid or methacrylic acid to provide carboxyl groups or an amino alkylated polymer to provide amino groups. US patent No. 4,654,267 describes the introduction of many such surface coatings. Alternatively, the support may carry other moieties for attachment, such as avidin or streptavidin (binding to biotin on the nucleotide sequence), DNA binding proteins (eg. the lac I repressor protein binding to a lac operator sequence which may be present in the starting molecule), or antibodies or antibody fragments (binding to haptens eg. digoxigenin on the nucleotide sequence). The streptavidin/biotin binding system is very commonly used in molecular biology, due to the relative ease with which biotin can be incorporated within nucleotide sequences, and indeed the commercial availability of biotin-labelled nucleotides, and thus biotin represents a particularly preferred means for immobilisation.

Where a solid phase amplification procedure is to be employed, the primer of the invention may additionally comprise a further mismatch(es) to create one or more additional restriction sites upstream of the potentially activating positions. Cleavage at such additional restriction sites may be used to detach the nucleotide sequences from the solid support in a quick and simple manner.

A preferred primer sequence according to the invention has the base sequence:

5' ACTGAATATA AACTTGTGGT CCATGGAGCT 3' and is designated herein primer 5K1.

The CCA is a mismatch corresponding to base 3 of codon 8 and bases 1 and 2 of codon 9 of the K-ras gene

and serves, as mentioned above, to introduce restriction sites overlapping activating positions in codons 12 and 13 of the wild-type K-ras gene. Primer 5K1 may, as mentioned above, be modified to enable introduction of a further restriction site upstream of the activating position. For example the modified primer designated 5K1-DraI having the base sequence

5' ACTGAATTTA AACTTGTGGT CCATGGAGCT 3'

contains an additional mismatch T corresponding to position 2 of codon 3 of the K-ras sequence and serves to introduce a DraI site for cleavage from the support. The primers may be biotinylated to facilitate the use of solid phase techniques.

For codon 61, a preferred primer may have the base sequence:

5'-TGTCTCTTGGATATTCTCGACACAGCAGCT-3'

The C is a mismatch corresponding to base 2 of codon 60 of the K-ras gene and serves, as mentioned above, to introduce a Bce 831 restriction site overlapping codon 61 of the wild-type K-ras gene.

The cleavage site for endonuclease Bce 83I is:

NNNNNNNNNNNNNNNNNNCTCAAG
NNNNNNNNNNNNNNNNNNGAGTTC

The primers may be modified, for example to create other, different or additional, restriction sites at upstream positions, or to create functionally equivalent analogue primer sequences, capable of functioning, as described above, to mediate the formation of the desired restriction sites.

Primers of the invention are used, as mentioned

above, in the in vitro amplification of K-ras sequences in the samples under investigation (i.e. in which activating K-ras mutations are to be detected) using, for example methods as described by Kahn et al., (Supra). Such a sample, which may comprise, for example, blood, serum, urine, expectorate, ascites or other biological fluids, tissue biopsies (which may be fresh or fixed) or even stool samples, optionally appropriately treated using standard techniques to release nucleic acids, will generally contain predominating amounts of wild-type K-ras sequences and minor amounts of mutant, activated, K-ras sequences. Thus for example the nucleic acid isolation technique of our co-pending British Patent Application No. 9323305.4 filed on 11 November 1993 may be used. This involves boiling the sample and allowing it to cool and condense on and within a high surface area solid support.

Any of the in vitro amplification techniques well known and described in the literature may be used. PCR and its modifications will however generally be the principal method of choice. In the case of classical PCR, two primers are of course required, hybridising to opposing strands of the target DNA. The primer of the invention will be used in this regard as the 5', or upstream, amplification primer, and a 3', or downstream, amplification primer may be selected according to choice. In this first amplification step, the choice of 3' primer is not especially critical, as long as it is capable of annealing to the template with sufficient specificity to enable specific amplification. Suitable 3' amplification primers include for example:

5' TTATCTGTAT CAAAGAATGG TCCTGCACCA 3' (3K1)
5' TATTAAAACA AGATTTAC 3' (3K3)

The sequences and positions of primers 5K1, 3K1 and 3K3 mentioned above, with respect to the wild-type human

K-*ras* gene are shown in Figure 2, which also shows the respective restriction endonuclease recognition sites which are created. Functionally equivalent modifications of such primer sequences may also be employed.

Modifications of the classical PCR method include, for example, the use of nested primers, in which an additional two "inner" primers are used, which "nest" or hybridise between the first "outer" primer pair. The use of four separate priming events results in increased specificity of the amplification reaction.

Other amplification techniques worthy of mention include Self-sustained Sequence Replication (SSR) and the Q-beta replicase amplification system.

In SSR, primers are used which carry polymerase binding sites permitting the action of reverse transcriptase to amplify target FNA or ssDNA.

In the Q-beta replicase system, an immobilised probe captures one strand of target DNA and is then caused to hybridise with an RNA probe which carries as a template region a tertiary structure known as MDV-1 for an RNA-directed RNA polymerase, normally Q-beta replicase.

As a result of this first amplification step, a population of K-*ras* fragments is created; amplified wild-type allele fragments contain the primer-mediated restriction sites, whereas mutant allele fragments do not. Subsequently, an aliquot of the amplification mixture is digested with the appropriate restriction enzyme under conventional conditions. The product may then be subjected to direct analysis for detection of the mutant K-*ras* alleles, for example using the RFLP analysis method of Kahn *et al*., 1990 (Supra), but more preferably will be subjected to further rounds of amplification for selective "enriched" amplification of the mutant sequences, for example following the method of Kahn *et al*., 1991 (Supra). In this step, the wild-

type sequences have been cleaved and hence are inaccessible to further amplification.

Following this, second, "enriching" amplification step the amplification mixture may be subjected to detection of the mutant K-ras sequence. This may take place by restriction endonuclease digestion and RFLP analysis using the procedure of Kahn et al., 1990 (Supra) or other detection methods. Particular mention may be made in this regard of the detection method known as "detection of immobilised amplified nucleic acids" or DIANA, which is a particularly advantageous technique to be used (see WO90/11369).

In the DIANA detection system, a further PCR amplification step is effected using nested primers, that is a first pair of primers to amplify the target nucleic acid in a first series of cycles, and a second pair of primers hybridising between the first primer pair in a second series of cycles. The inner primers used in the second cycle carry, respectively, means for immobilisation to permit capture of the amplified DNA and a label or means for attachment of a label to permit recognition. The means for immobilisation may, for example, be a hapten such as biotin or digoxigenin while the means for attachment of a signal may include a different hapten or, in a preferred embodiment, a 5'-non-hybridising DNA sequence which is capable of binding to a DNA-binding protein (e.g. the lac operator) carrying an appropriate label. The immobilisation means may also be attached via a 5'-non-hybridising DNA sequence.

As a further check on accuracy, or as the primary detection method, the amplified fragments may be subjected to sequence analysis to verify the mutation, using known sequencing techniques. As mentioned above, this has advantages from a diagnostic point of view, as important differential diagnostic information may be provided.

Conveniently, the final amplification prior to analysis may take place by cycle sequencing. This has the advantage of yielding the base substitution information directly. Such a step would be particularly advantageous for an automated process.

In a further aspect, the present invention thus provides use of an oligonucleotide primer sequence according to the invention as defined above, in an in vitro amplification-based method for detection of activating mutations in codons 12 and/or 13 and/or 61 of the K-ras oncogene.

Viewed from a further aspect, the invention can also be seen to provide a method for detecting activating mutations in codons 12 and/or 13 and/or 61 of the K-ras oncogene, said method comprising subjecting a sample containing the target K-ras DNA to be detected to one or more cycles of in vitro amplification using as an amplification primer, an oligonucleotide primer sequence according to the invention as defined above, followed by restriction endonuclease digestion of wild-type K-ras sequences, using BstX I and/or Xcm I and/or Bce 83I, optionally followed by one or more further cycles of in vitro amplification whereby mutant K-ras sequences containing said activating mutations are enriched, and detecting the said amplified mutant K-ras sequences obtained.

It is preferable, in carrying out the amplification steps according to the invention to introduce one or more additional "control" restriction sites in the amplified fragments. This may be achieved by primer-mediation, using the 3' amplification primer in a manner analogous to that described for the 5' primers of the invention above. Thus, mismatches present in the 3' amplification primer may be used to create the desired control restriction sites (which will correspond to the sites introduced by the 5' primer of the invention). This provides a system to monitor the fidelity of

restriction enzyme cleavage.

Thus for example, the following 3' amplification primer may be used: 5' GAATGGTCCT CCACCAGTA TATGGATATT A 5' (designated herein primer 3K2). The mismatches at positions C and G respectively serve to create BstX I and Xcm restriction sites. Primer 3K2 and its relative position is also shown in Figure 2.

Where an additional, selective "enriching" amplification step is employed according to the invention, the "modified" internal control 3' primer, will generally only need to be used in the enriching amplification step.

It may also in certain cases be desirable to introduce further modifications to the method, in order for example to enhance specificity and/or sensitivity. Thus it may sometimes occur, that not all of the wild-type K-ras sequences will be digested in the enzyme cleavage step, and that some undigested wild-type sequences may remain. Also, mutated and wild-type strands may reanneal to form heteroduplexes which are not recognised by the restriction enzymes. To enhance specificity of the subsequent "enriching" selective amplification stage, additional amplification and restriction cleavage steps may be employed, using for example nested primers to further enhance specificity. This will be described in more detail in the Examples below, and is illustrated schematically in Figure 3. It has been shown that by using two restriction endonuclease cutting steps, sensitivity may be improved up to 1:100,000.

Further modifications include as mentioned above the use of solid supports to immobilise the amplification products. In this case, one or both of the amplification primers may be-provided with means for immobilisation e.g. biotin or haptens etc as described above. The use of such a solid phase system is advantageous in that it is cleaner, more efficient and

allows the whole process to be carried out in a single tube, thereby minimising losses.

In all the amplification stages, the amplification cycles may be performed using standard conditions which are well known in the art. In order to enhance specificity and sensitivity, it is preferable however to keep the total number of cycles to a minimum and to hybridise the amplification primers at high stringency.

The oligonucleotides according to the invention may be synthesised by known techniques using conventional machine synthesisers such as the Cyclone DNA synthesiser (Biosearch Inc.).

The invention also extends to kits for detection of activating mutations in codons 12 and/or 13 and/or 61 of the K-*ras* oncogenes, comprising at least one oligonucleotide primer sequence according to the invention. Such kits will normally also contain such additional components as:

(a) for PCR, a polymerase and at least one other oligonucleotide primer; the oligonucleotides both being DNA based and hybridising to opposite strands of the target DNA;

(b) for DIANA, a polymerase and PCR oligonucleotide primers according to the invention provided with means for immobilisation and means for labelling;

(c) for 3SR, a reverse transcriptase and a further DNA oligonucleotides primer, both oligonucleotides being provided with a polymerase binding site;

(d) for Q-beta replicase amplification, an RNA directed RNA polymerase and an RNA probe with a 5'-MDV-1 structure, the capture oligonucleotide being immobilised or permitting immobilisation.

In all the above kits, nucleotide bases will normally be supplied together with appropriate buffers.

The following Examples are given by way of illustration only with reference to the following Figures in which:

Figure 1 shows the nucleotide and corresponding amino acid sequence of the human cellular proto-oncogene K-ras (c-ki-ras2). Mutating hot spots are underlined;

Figure 2 shows the sequences of the primers used in the Examples and their positions with respect to the wild-type K-ras gene. The cleavage sites of the restriction endonucleases BstXI and XcmI are also shown;

Figure 3 shows a schematic representation of the steps involved in carrying out the K-ras mutation detection procedure of the present invention as described in Examples 1 and 2;

Figure 4 shows the results of electrophoresis of the amplification products of Example 2 in a 4% agarose gel containing ethidium bromide and analysed under UV light; and

Figure 5 shows the results of sequence analysis of the products of Example 2, 6% polyacrylamide gel. Panel 3A shows the sequence of the mutated products obtained from the needle biopsy (sample 3A); Panel 3B shows the sequence obtained from the same sample using the same procedure but without adding endonuclease (BstXI) in the two intermediate destructions of non-mutated amplification product.

Example 1

This sets out a general description of the performance of the method of the invention, to detect mutation in codon 12 of the K-ras gene, using a primer according to the invention, and is illustrated schematically in Figure 3.

1: DNA may be isolated from different types of sample (expectorate, faeces, fixed tissue, urine, ascites, blood, etc.).

2: A small amount of sample is mixed in a salt-buffer containing a biotinylated oligonucleotide probe for the gene under study. The tube is then heated and vortexed to release and denature DNA-strands. In this example we have a mix of normal DNA (in excess) and DNA with a point mutation (G→A) in the second base of codon 12 of the K-ras gene.

3: The solution is cooled, and the probe binds to the denaturated DNA.

4: Magnetic streptavidin coated beads are added to solution, DNA binds to the beads both unspecifically and by the streptavidin-biotin binding (where there is a biotinylated primer).

5: Beads with bound DNA are isolated from contaminating debris and inhibitors by magnetic separation, and added as template to a PCR amplification.

6: Amplification is performed using a modified 5'primer (5K1) and an ordinary 3'primer (3K1).

7: An amplification product containing the induced modifications is produced. This product is then used in two parallel procedures which differ only in the type of endonuclease used.

8a: The amplification product is incubated with a endonuclease (BstX 1) specific for the sequence CCANNNNNNGTT (N = G, T, A or C).

8b: The amplification product is incubated with a

endonuclease (Xcm 1) specific for the sequence CCANNNNNNNNNGTT (N = G, T, A or C).

9a: BstX 1 cuts the product with a normal codon 12 but not the mutated product. For several reasons there may not be a complete cutting of the normal sequence.

9b: Xcm 1 cuts both the normal and mutated product since there is no mutation in codon 13. For several reasons there may not be a complete cutting of the product.

10a/b-13a/b: The digested product is used as template in an amplification identical to step 6. The mutated product is preferentially amplified because most of the normal product has been cut by the enzymes. The

endonuclease digestion is repeated to increase sensitivity.

14a/b: The digested product is used as template in an amplification using a biotinylated 5'primer (5K1-bio) and a modified 3'primer (3K2) containing both of the two restriction sites.

15a/b: An amplification product containing the modifications is produced.

16a/b: The products are digested with their respective endonucleases.

17a: The mutated product is cut only at the control site introduced by 3K2, while the normal product is cut at both sites.

17b: All of the product is cut at both sites since there is no mutation in codon 13.

18a: The figure illustrates how different ratios of mutated vs. normal in the sample will look on an electrophoresis of the digested end-product. A 133 bp band indicate mutation, normal products result in a 107 bp band and undigested products are seen as a band at 152 bp.

18b: All of the product from a sample with mutation in codon 12 are cut by Xcm 1. This enzyme is specific for the amplification product carrying wild type codon 13.

19a/b: From the electrophoresis we can determine if there is a mutation and in what codon. To identify the type of base substitution, the biotinylated strand of the product is sequenced by solid-phase sequencing.

Example 2

Detection of K-ras mutation in formalin-fixed paraffin-embedded needle-biopsy from pancreatic cancer

This Example was performed using the general procedure set out in Example 1.

Sample and controls:

1: Colon-cancer cell-line SW480 with known K-ras codon 12 mutation (GTT) and no normal allele.

2: Formalin-fixed paraffin-embedded tissue from normal colonic mucosa.

3: Formalin-fixed paraffin-embedded needle-biopsy (1mm x 10mm) from pancreatic cancer.

4: Distilled water (control without DNA).

DNA preparation:

From cell-line:

DNA from SW480 is extracted (phenol/chloroform) and quantified using standard methods. 1 μg of this DNA is used as positive control of mutation.

From paraffin blocks:

Two 5 μm thick slides are cut of from each of the blocks. The microtom blade is washed twice in xylol and once in ethanol before each cutting.

The slides are added to 500 μl micro-centrifuge tubes containing:

Bind & Wash buffer (Dynal, Norway), 400 μl:
Tris-HCl 10 mM, pH 7.5
EDTA 1mM
NaCl 2.0 M
5K1-Bio 3 pmol

The tubes are incubated at 94°C for 5-10 minutes in a thermal cycler, and the contents mixed twice on a vortex-mixer for 20 seconds during this incubation.

When the tubes has been cooled to ambient temperature (for about 3 minutes), the liquid phase is pipetted off and mixed with 20 μg of streptavidin coated paramagnetic beads (Dynabeads© M-280 streptavidin, Dynal, Norway).

The mixture is left at ambient temperature for 15 minutes.

The beads are isolated by magnetic separation (Dynal MPC©-E, Dynal, Norway), and used as template to the

amplification reaction.

Amplification:

Three (A1, A2 and B) serial PCRs with two intermediate destructions of non-mutated alleles by a specific endonuclease (in this case BstX 1) are performed. To avoid miss-priming a "hot-start" PCR-method is performed (i.e. the amplification reaction is not started until the reaction-mix reaches a certain temperature). This is accomplished by dividing essential reagents by a layer of wax (Para Clean II (melting range 55-57°C), Klinipath, Zevenaar, Netherlands) which melts at higher temperatures.

PCR amplifications was performed in a total volume of 50 μl:

dNTP 0.025 mM each

potassium chloride 50 mM

magnesium chloride 1.5 mM

Tris-HCI 10 mM pH 8.4

gelatin 0.01%

primers 0.2 μM each

Taq polymerase 2.5 units (AmpliTaq® DNA Polymerase, Perkin Elmer, CT, USA)

The wax (17 μl) divides the reaction-mix in two compartments of equal volume. Only the lower compartment contains dNTP and primers, and polymerase and template are added above the wax-layer.

Thermal-cycling is performed in 200 μl MicroAmp™ reaction tubes with the GenAmp™ PCR System 9600 (Perkin-Elmer, CT, USA):

One cycle consists of 30 sec denaturation at 94°C, 1 min. annealing at 50°C and 2 min. elongation at 72°C. After the last cycle the tubes are kept for 8 min. at the elongation temperature.

Destruction of non-mutated amplification product:

5 μl of the amplification product is digested with restriction endonuclease BstX 1 (New England Biolabs, MA, USA) in a total volume of 20 μl at conditions recommended by the supplier.

5 μl of the digested solution is used as template in the proceeding amplification step.

Amplification/Digestion procedure:

The first PCR (A1) consists of 15 cycles with 5K1 and 3K1 as primers and genomic DNA as template.

The amplification product is then digested with BstX 1 (A samples, B samples follow the same procedure but without the enzyme (BstX 1)) to destroy product that is not mutated in codon 12 for further amplification.

The digested product is used as template for 15 more cycles with 5K1 and 3K1 as primers (PCR A2).

A second BstX 1 digestion (A samples, B samples follow the same procedure but without the enzyme (BstX 1)) is performed to increase sensitivity.

The digested product is used as template for 35 cycles (PCR

B) with a biotinylated primer (5K1-Bio) and a primer introducing control restriction-sites (3K2).

RFLP analysis:

Ten μl of the amplification product of PCR B is digested with BstX 1 in a total volume of 20 μl.

The digested amplification product is subjected to electrophoresis in a 4% agarose gel containing ethydium bromide and analyzed in UV-light (Figure 4).

Mutated product is identified as a 133 bp band. Non-mutated samples show up as 107 bp bands if they are not completely eliminated by the procedure. Undigested product is found as 152 bp fragments.

Solid-phase sequencing:

The oligonucleotide sequence of the amplification product is identified by solid-phase sequencing using streptavidin coated paramagnetic beads (Dynabeads© M-280 streptavidin, Dynal, Norway) as solid-support.

The biotinylated strand of the amplification product (PCR B) is isolated as described by the supplier of the beads (Technical handbook, Molecular Biology, 1st Ed., Dynal, Norway).

The sequencing reaction is performed as described by the supplier of the kit (Sequenase®, Version 2.0, United States Biochemical, Ohio, USA) with 3K3 as the sequencing primer.

The sequence of the mutated product obtained from the needle-biopsy (sample 3A) is shown in Figure 5. The right sequence (3B) is obtained from the same sample using the

same procedure, but without adding endonuclease (BstX 1) in the two intermediate destructions of non-mutated amplification product. This illustrates how a weak non-informative mutated band is enhanced, and the background of the normal allele is removed.

An identical procedure using endonuclease Xcm 1 instead of BstX 1 allows detection of mutations in codon 13.

Claims:

1. An oligonucleotide primer sequence for use in *in vitro* amplification, characterised in that said primer sequence is capable of creating a *BstX* I restriction site overlapping codon 12 and/or an *Xcm* I restriction site overlapping codon 13 of the wild-type K-*ras* oncogene.

2. An oligonucleotide primer sequence as claimed in claim 1 wherein said sequence contains a CCA substitution corresponding to the final base of codon 8 and the first two bases of codon 9 of the K-*ras* gene.

3. An oligonucleotide primer sequence as claimed in claim 2 wherein said sequence comprises the base sequence:

5' ACTGAATATA AACTTGTGGT CCATGGAGCT 3'.

4. An oligonucleotide primer sequence for use in *in vitro* amplification, characterised in that said primer sequence is capable of creating a *Bce* 83I restriction site overlapping codon 61 of the wild-type K-*ras* oncogene.

5. An oligonucleotide primer sequence as claimed in claim 4, wherein said sequence contains a C substitution corresponding to the second base of codon 60 of the K-*ras* gene.

6. An oligonucleotide primer sequence as claimed in claim 5 wherein said sequence comprises the base sequence:

5' TGTCTCTTGG ATATTCTCGA CACAGCAGCT 3'.

7. An oligonucleotide primer sequence as claimed in any one of claims 1 to 6 wherein said primer sequence is capable of creating one or more additional restriction sites.

8. An oligonucleotide primer sequence as claimed in claim 7 wherein the additional restriction site is a *Dra*I

restriction site upstream of the restriction site overlapping codon 12 and/or codon 13 of the wild-type K-ras oncogene.

9. An oligonucleotide primer sequence as claimed in claim 8 wherein said sequence comprises the base sequence:

5' ACTGAATTTA AACTTGTGGT CCATGGAGCT 3'.

10. An oligonucleotide primer sequence as claimed in any one of claims 1 to 9 wherein the sequence is 22 to 30 nucleotides long.

11. Use of an oligonucleotide primer sequence as claimed in any one of claims 1 to 10 in an in vitro amplification-based method for detection of activating mutations in codons 12 and/or 13 and/or 61 of the K-ras oncogene.

12. A method of detecting activating mutations in codons 12 and/or 13 and/or 61 of the K-ras oncogene, said method comprising subjecting a sample containing the target K-ras DNA to be detected to one or more cycles of in vitro amplification using as an amplification primer, an oligonucleotide primer sequence as claimed in any one of claims 1 to 10, followed by restriction endonuclease digestion of wild-type K-ras sequences, using BstX I and/or Xcm I and/or Bce 83I and detecting the said amplified mutant K-ras sequences obtained.

13. A method as claimed in claim 12 wherein restriction endonuclease digestion is followed by one or more further cycles of in vitro amplification whereby mutant K-ras sequences containing said activating mutations are enriched.

14. A method as claimed in claim 12 or 13 wherein an additional primer is used for amplification.

15. A method as claimed in claim 14 wherein the sequence of the additional primer is selected from:

5' TTATCTGTAT CAAAGAATGG TCCTGCACCA 3' and

5' TATTAAAACA AGATTTAC 3'.

16. A method as claimed in claim 14 wherein said additional primer is capable of creating at least one restriction site.

17. A method as claimed in claim 16 wherein said additional primer is capable of creating a BstX I and Xcm I restriction sites.

18. A method as claimed in claim 17 wherein said additional primer has the sequence

5' GAATGGTCCT CCACCAGTA TATGGATATT A 5'.

19. A method as claimed in any one of claims 12 to 18 wherein PCR is used for amplification.

20. A kit for the detection of activating mutations in codons 12 and/or 13 and/or 61 of the K-ras oncogenes, comprising at least one oligonucleotide primer sequence as claimed in any one of claims 1 to 10.

21. A kit as claimed in claim 20 wherein additional components for amplification are provided.

22. A kit as claimed in claim 21 wherein said amplification is performed using

a) PCR and the kit additionally comprises a polymerase and at least one other oligonucleotide primer sequence; the oligonucleotides both being DNA based and hybridising to opposite strands of the target DNA; or

b) DIANA and the kit additionally comprises a polymerase and PCR oligonucleotide primer sequences as claimed in any one of claims 1 to 10 provided with means for immobilisation and means for labelling; or

c) 3SR and the kit additionally comprises a reverse transcriptase and a further DNA oligonucleotides primer sequence, both oligonucleotides being provided with a polymerase binding site; or

d) Q-beta replicase and the kit additionally comprises an RNA directed RNA polymerase and an RNA probe with a 5'-MDV-1 structure, the capture oligonucleotide being immobilised or permitting immobilisation.

FIG. 1

CCGCGGTCCGGTCCCGCTCCGGGTCAGAATTGGCGGCTGCGGGGACAGCCTTGCGGCTAGGCAGGGGGCGGGCCGCCGCGTGGG
TCCGGCAGTCCCTCCTCCCGCCAAGGCGCCGCCCAGACCCGCTCTCCAGCCGGCCCGGCTCGCCACCCTAGACCCCCCCAGCC
ACCCCTTCCTCCGCCGGCCCGGCCCCCGCTCCTCCCCCGCCGGCCCGGCCCGGCCCCCTCCTTCTCCCCGCCGGCGCTCGCTGC
CTCCCCCTCTTCCCTCTTCCCACACCGCCCTCAGCCGCTCCCTCTCGTACGCCCGTCTGAAGAAGAATCGAGCGCGGAACGCA
TCGATAGCTCTGCCCTCTGCGGCCGCCCGGCCCCGAACTCATCGGTGTGCTCGGAGCTCGATTTTCCTAGGCGGCGGCCGCGGC
GGCGGAGGCAGCAGCGGCGGCGGCAGTGGCGGCGGCGAAGGTGGCGGCGGCTCGGCCAGTACTCCCGGCCCCCGCCATTTCGG
ACTGGGAGCGAGCGCGGCGCAGGCACTGAAGGCGGCGGCGGGGCCAGAGGCTCAGCGGCTCCCAGGTGCGGGAGAGAG*GTACGG*
←EXON 0
AGCGGACCACCCCTCCTGGCCCCTGCCCGGGTCCCGACCCTCTTTGCCGGCGCCGGGCGGGGCCGGCGGCGAGTGAATGAAT
TAGGGGTCCCCGGAGGGGCGGTGGGGGCGCGGGCGCGGGGTCGGGGCGGGCTGGGTGAGAGGGGTCTGCAG>>>GTACTGGT
GGAGTATTTGATAGTGTATTAACCTTATGTGTGACATGTTCTAATATAGTCACATTTTCATTATTTTTATTATAAG **GCCTGC**
EXON 1 →

Met Thr Glu Tyr Lys Leu Val Val Val Gly Ala <u>Gly</u>(12) <u>Gly</u>(13) Val Gly Lys Ser Ala Leu Thr
TGAAA ATG ACT GAA TAT AAA CTT GTG GTA GTT GGA GCT <u>GGT</u> <u>GGC</u> GTA GGC AAG AGT GCC TTG ACG

Ile Gln Leu Ile Gln Asn His Phe Val Asp Glu Tyr Asp Pro Thr Ile Glu
ATA CAG CTA ATT CAG AAT CAT TTT GTG GAC GAA TAT GAT CCA ACA ATA GAG *GTAAATCTTGTTT*
←EXON 1

TAATATGCATATTACTGGTGCAGGACCATTCTTTGATACAGATAAAGGTTTCTCTGACCATTTTCATGAGT>>>ATCACCATTT
TACATTCCCACCAGCAATGCACAAAGATTTCAGTGTCTGTATCCTTGCTAACACTTATTTTCCATTTTTTGAGTTTTTTTGTT
TTGTTTTTTTAATAATAGCCAATCCTAATGGGTATGTGGTAGCATCTCATGGTTTTGATTTTATTTTCCTGACTATTGATGATG
TTGAGCATCTTTTCAGGTGCTTAGTGGCCATTTGTCCGTCATCTTTGGAGCAGGAACAATGTCTTTTCAAGTCCTTTGCCCAT
TTTTAAATTGAATTTTTTGTTGTTGAGTTGTATATAACACCTTTTTTGAAGTAAAAGGTGCACTGTAATAATCCAGACTGTGTT

Asp Ser Tyr Arg Lys Gln Val Val Ile Asp Gly Glu Thr Cys Leu Leu Asp
TCTCCCTTCTCAG **GAT TCC TAC AGG AAG CAA GTA GTA ATT GAT GGA GAA ACC TGT CTC TTG GAT**
EXON 2 →

Ile Leu Asp Thr Ala Gly <u>Gln</u>(61) Glu Glu Tyr Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly
ATT CTC GAC ACA GCA GGT <u>CAA</u> GAG GAG TAC AGT GCA ATG AGG GAC CAG TAC ATG AGG ACT GGG

Glu Gly Phe Leu Cys Val Phe Ala Ile Asn Asn Thr Lys Ser Phe Glu Asp Ile His His Tyr
GAG GGC TTT CTT TGT GTA TTT GCC ATA AAT AAT ACT AAA TCA TTT GAA GAT ATT CAC CAT TAT
←EX

Ar
AG *GTGGGTTTAAATTGAATATAATAAGCTGACATTAAGGAGTAATTATAGTTTTTATTTTTTGAGTCTTTGCTAATGCCATGC*
ON 2
ATATAATATTTAATAAAAATTTTTAAATAATGTTTATGAGGTAGGTAATATCCCTGTTTTATAAATGAAGTTCTTGGGGGATT
AGAGCAGTGGAGTAACTTGCTCCAGACTGCATCGGTAGTGGTGGTGCTGGGATTGAAACCTAGGCCTGTTTGACTCCACAGCCT
TCTGT>>>TCTAGAATTTTTCAGTAGTTTCTGTTTTACTATTATGATCTACCTGCATATTAACCTATTAGGTTATAGTTTTAC
TATACTTCTAGGTATTTGATCTTTTGAGAGAGATACAAGGTTTCTGTTTAAAAAGGTAAAGAAACAAAATAACTAGTAGAAGAA
GGAAGGAAAATTTGGTGTAGTGGAAACTAGGAATTACATTGTTTTCTTTCAGCCAAATTTTATGACAAAAGTTGTGGACAGGT

```
                                                        g Glu Gln Ile Lys Arg Val
TTTGAAAGATATTTGTGTTACTAATGACTGTGCTATAACTTTTTTTTCTTTCCCAG A GAA CAA ATT AAA AGA GTT
                                                         EXON 3 →

Lys Asp Ser Glu Asp Val Pro Met Val Leu Val Gly Asn Lys Cys Asp Leu Pro Ser Arg Thr
AAG GAC TCT GAA GAT GTA CCT ATG GTC CTA GTA GGA AAT AAA TGT GAT TTG CCT TCT AGA ACA

Val Asp Thr Lys Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Phe Ile Gly Thr Ser
GTA GAC ACA AAA CAG GCT CAG GAC TTA GCA AGA AGT TAT GGA ATT CCT TTT ATT GAA ACA TCA

Ala Lys Thr Arg Gln
GCA AAG ACA AGA CAG GTAAGTAACACTGAAATAAATACAGATCTGTTTTCTGCAAAATCATAACTGTTATGTCATTTA
                ← EXON 3
ATATATCAGTTTTTCTCTCAATTATGCTATACTAGGAAATAAAACAATATTTAGTAAATGTTTTTGTCTCTTGAGAGGGCATTG
CTTCTTAATC>>ACAGAAGACCCAGTCTCAGCTTCACTTGTATACCCTGGAAATAGACTGAAAGGTGTTAAAATTTAAAATAA
AACTCAAGGTTCCAGTTTCTTGACTCACCTTTGAGATTCTTTTATGTTTTTGTTGTTTTTAACAAAGGTTTCACGTCCATATT
TTACCATTTTTCTTCTCATTCTCCCCTGGAGGAGGGTGTGGGAATCGATAGTATATAAATCACTTTTTTCCTAAGTCAAAGAA
GTAATTTAAAGCTAACTTCAGTTTAGGCTTTAATTCCAGGACTAGCAAACTAAAATGGTTGCATTAATTGACAAACAGATGCTA
ATACCTGTGTTTAGGCTTGTCATAATCTCTCCTAATTCCTAATTTAAAAATTTTAAAATTTAATTCCATTAGAAAACAAAACC
GACTTTTAAGAACAAACCAGGATTCTAGCCCATATTTTAAAACTGCATCCTCAGTTTTATTCAAACAGTCTGATGTCTGTTTAA
AAAAAAAAAAATCTCAAGCTCATAATCTCAAACTTCTTGCACATGGCTTTCCCAGTAAATTACTCTTACCAATGCAACAGACT

                            Arg Val Glu Asp Ala Phe Tyr Thr Leu Val Arg Glu Ile Arg
TTAAAGAAGTTGTGTTTTACAATGCAG AGA GTG GAG GAT GCT TTT TAT ACA TTG GTG AGA GAG ATC CGA
                            EXON 4A →

Gln Tyr Arg Leu Lys Lys Ile Ser Lys Glu Glu Lys Thr Pro Gly Cys Val Lys Ile Lys Lys
CAA TAC AGA TTG AAA AAA ATC AGC AAA GAA GAA AAG ACT CCT GGC TGT GTG AAA ATT AAA AAA

Cys Ile Ile Met OC
TGC ATT ATA ATG TAA TCTG GTAAGTTTAAGTTCAGCACATTAATTTTGGCAGAAAGCAGATGTCTTTTAAAGGTAACA
                ← EXON 4A
AGGTGGCAACCACTTTAGAACTACTTAGGTGTAGTATTCTAACTTGAAGTATTAAAAGATAAGAAACTTGTTTCCATAATTAG
T>>>GAATTCTAAAAGTCCTAATATATGTAATATATATTCAGTTGCCTGAAGAGAAACATAAAGAATCCTTTCTTAATATTTTT
                                                                               Gly
TCCATTAATGAAATTTGTTACCTGTACACATGAAGCCATCGTATATATTCACATTTTAATACTTTTTATGTATTTCAG GGT
                                                                               EXON 4B

Val Asp Asp Ala Phe Tyr Thr Leu Val Arg Glu Ile Arg Lys His Lys Glu Lys Met Ser Lys
GTT GAT GAT GCC TTC TAT ACA TTA GTT CGA GAA ATT CGA AAA CAT AAA GAA AAG ATG AGC AAA

Asp Gly Lys Lys Lys Lys Lys Lys Ser Lys Thr Lys Cys Val Ile Met OC
GAT GGT AAA AAG AAG AAA AAG AAG TCA AAG ACA AAG TGT GTA ATT ATG TAA ATACAATTTGTACTT

TTTTCTTAAGGCATACTAGTACAAG TGGTAATTTTTGTACATTACACTAAATTATTAGCATTTGTTTTAGCATTACCTAATTT
                   ← EXON 4B
TTTTCCTGCTCCATGCAGACTGTTAGCTTTTACCTTAAATGCTTATTTTAAAATGACAGTGGAAGTTTTTTTTTCCTCGAAGT
GCCAGTATTCCCAGAGTTTTGGTTTTTGAACTAGCAATGCCTGTGAAAAAGAAACTGAATACCTAAGATTTCTGTCTTGGGGTT
TTTGGTGCATGCAGTTGATTACTTCTTATTTTTCTTACCAAGTGTGAATGTTGGTGTGAAACAAATTAATGAAGCTT
```

FIG. 1 CONT'D

FIG. 2

```
                                 Xcm I
                              CCA---------TGG

                                BstX I
                              CCA------TGG

               5K1
     [------------------CCA------>

TGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTG>>>
ACTTTTACTGACTTATATTTGAACACCATCAACCTCGACCACCGCATCCGTTCTCACGGAACTGCTATGTCGATTAAGTCTTAGTAAAAC<<<

>>>TGGACGAATATGATCCAACAATAGAGGTAAATCTTGTTTTAATATGCATATTACTGGTGCAGGACCATTCTTTGATACAGATAAAGGTTT
<<<ACCTGCTTATACTAGGTTGTTATCTCCATTTAGAACAAAATTATACGTATAATGACCACGTCCTGGTAAGAAACTATGTCTATTTCCAAA

                                                                                3K1
                                                              <----------------------------]

                                                              3K2
                                                   <-----G------------C---------]

                                                         Xcm I
                                                      CCA---------TGG

                                                        BstX I
                                                      CCA------TGG

                                        3K3
                                      <----------------]
```

Primers:

5K1: ACTGAATATAAACTTGTGGTCCATGGAGCT

5K1-Bio: Biotin-ACTGAATATAAACTTGTGGTCCATGGAGCT

3K1: TTATCTGTATCAAAGAATGGTCCTGCACCA

3K2: GAATGGTCCTCCACCAGTAATATGGATATTA

3K3: TATTAAAACAAGATTTAC

Endonucleases:

```
         C C A N N N N N|N T G G                C C A N N N N N|N N N N T G G
BstX I:  G G T N|N N N N N A C C       Xcm I:  G G T N N N N|N N N N N N A C C
```

FIG. 3

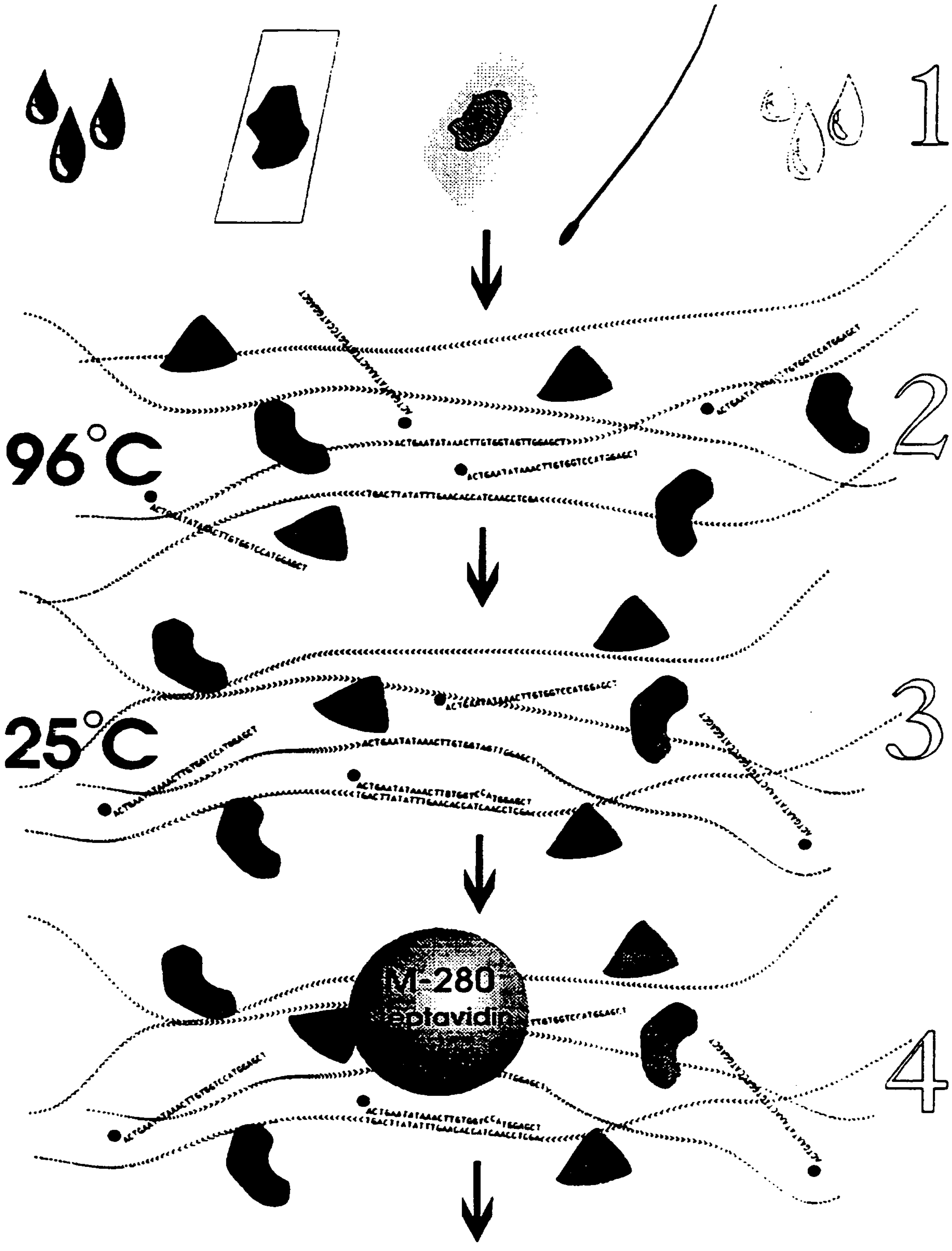


FIG. 3 CONT'D

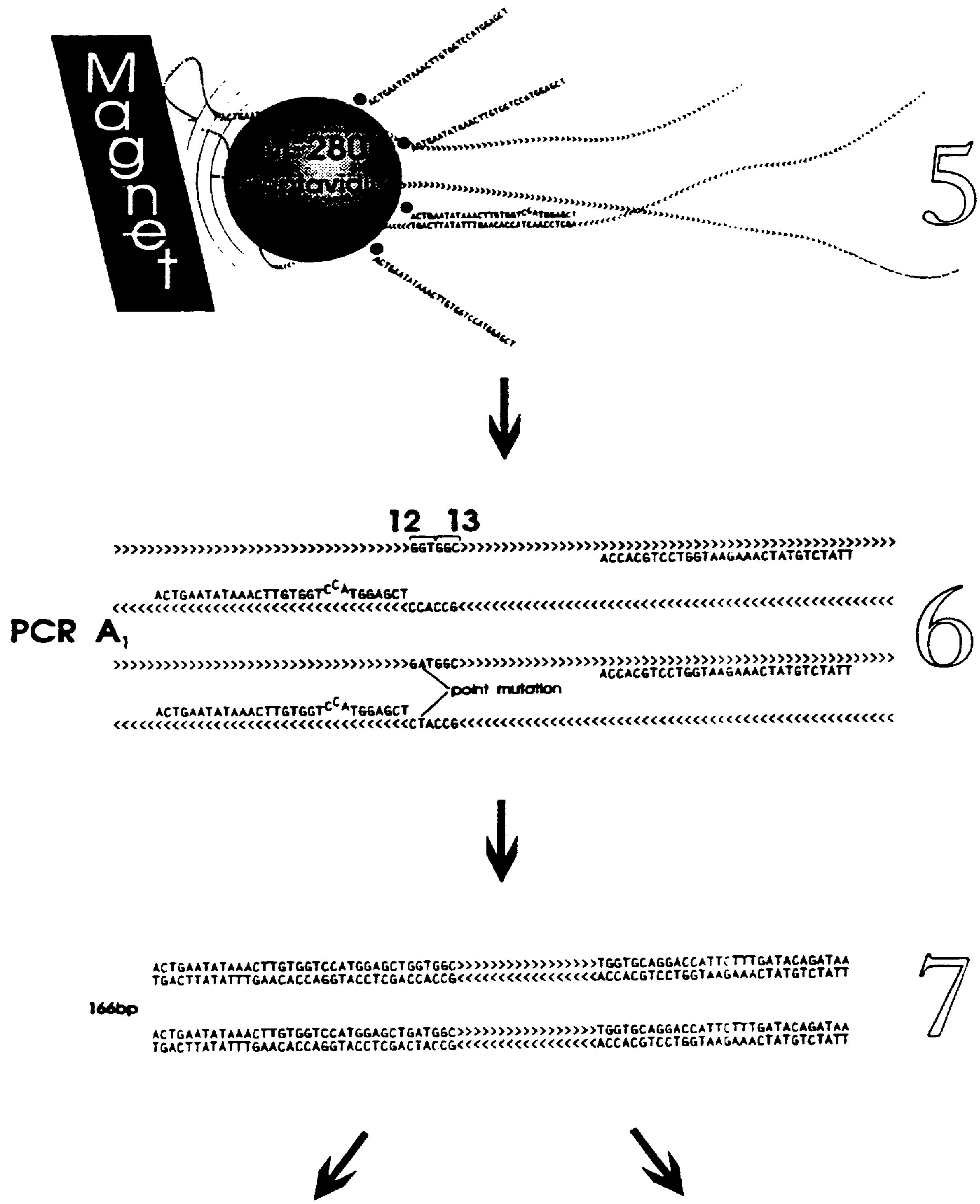

Magnet
5
12 13
GGTGGC
ACCACGTCCTGGTAAGAAACTATGTCTATT
ACTGAATATAAACTTGTGGTCCATGGAGCT
CCACCG
PCR A1
6
GATGGC
ACCACGTCCTGGTAAGAAACTATGTCTATT
point mutation
ACTGAATATAAACTTGTGGTCCATGGAGCT
CTACCG
ACTGAATATAAACTTGTGGTCCATGGAGCTGGTGGC
TGGTGCAGGACCATTCTTTGATACAGATAA
TGACTTATATTTGAACACCAGGTACCTCGACCACCG
ACCACGTCCTGGTAAGAAACTATGTCTATT
166bp
ACTGAATATAAACTTGTGGTCCATGGAGCTGATGGC
TGGTGCAGGACCATTCTTTGATACAGATAA
TGACTTATATTTGAACACCAGGTACCTCGACTACCG
ACCACGTCCTGGTAAGAAACTATGTCTATT
7

FIG. 3CONT'D

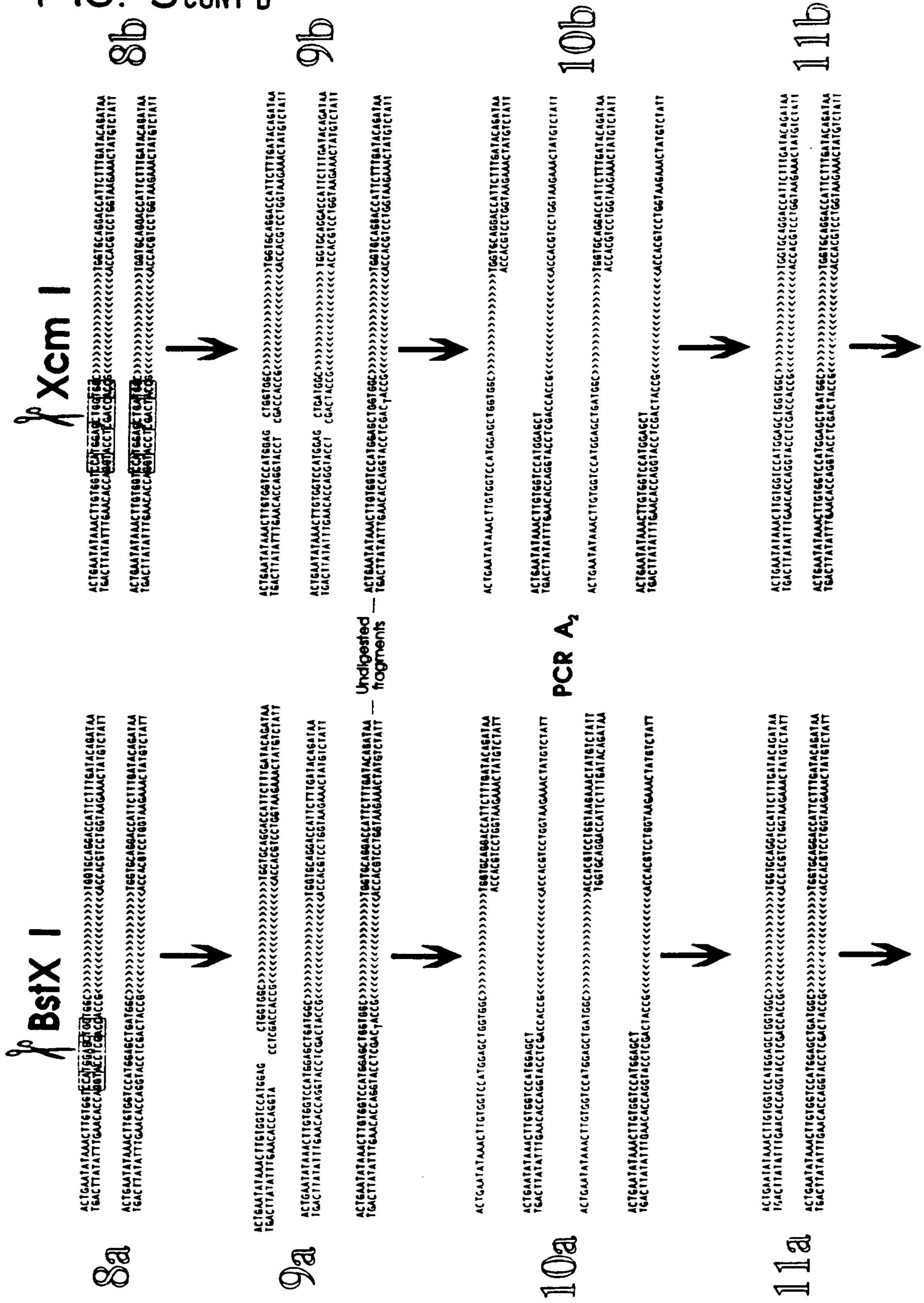
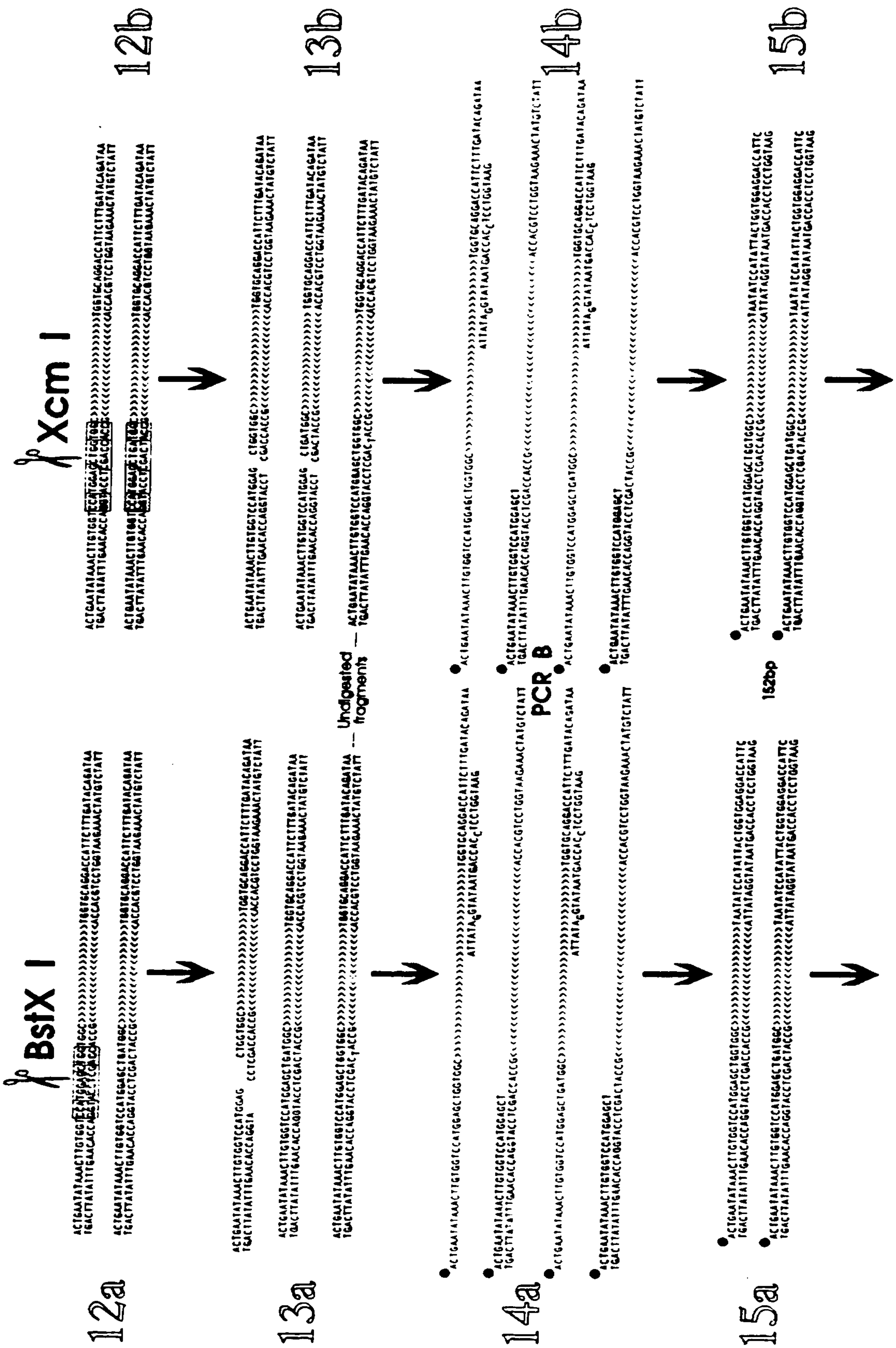
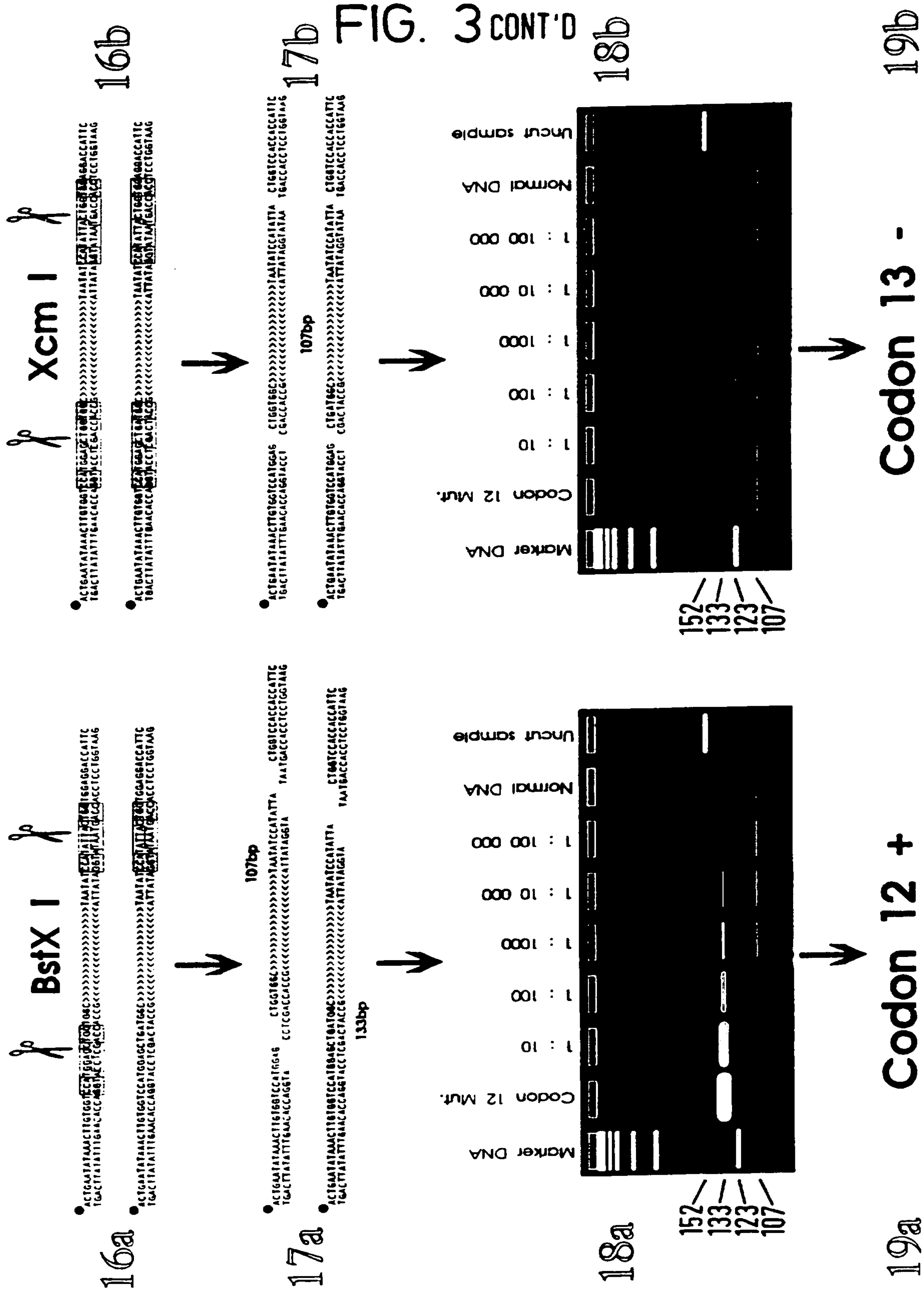

BstX I
Xcm I
8a
8b
9a
9b
10a
10b
11a
11b
Undigested fragments
PCR A2

FIG. 3 CONT'D


BstX I
Xcm I
12a
12b
13a
13b
14a
14b
15a
15b
Undigested fragments
PCR B
152bp

8/10

FIG. 3 CONT'D



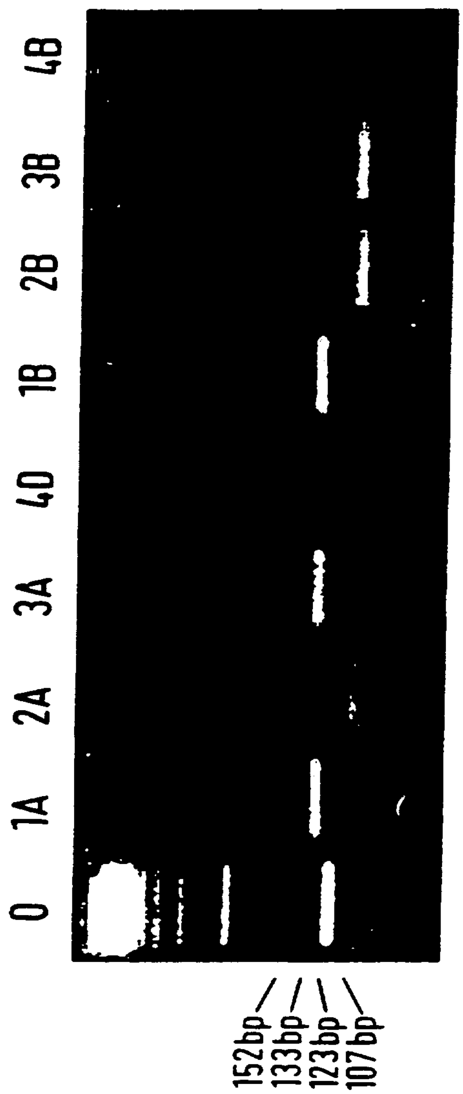


FIG. 4

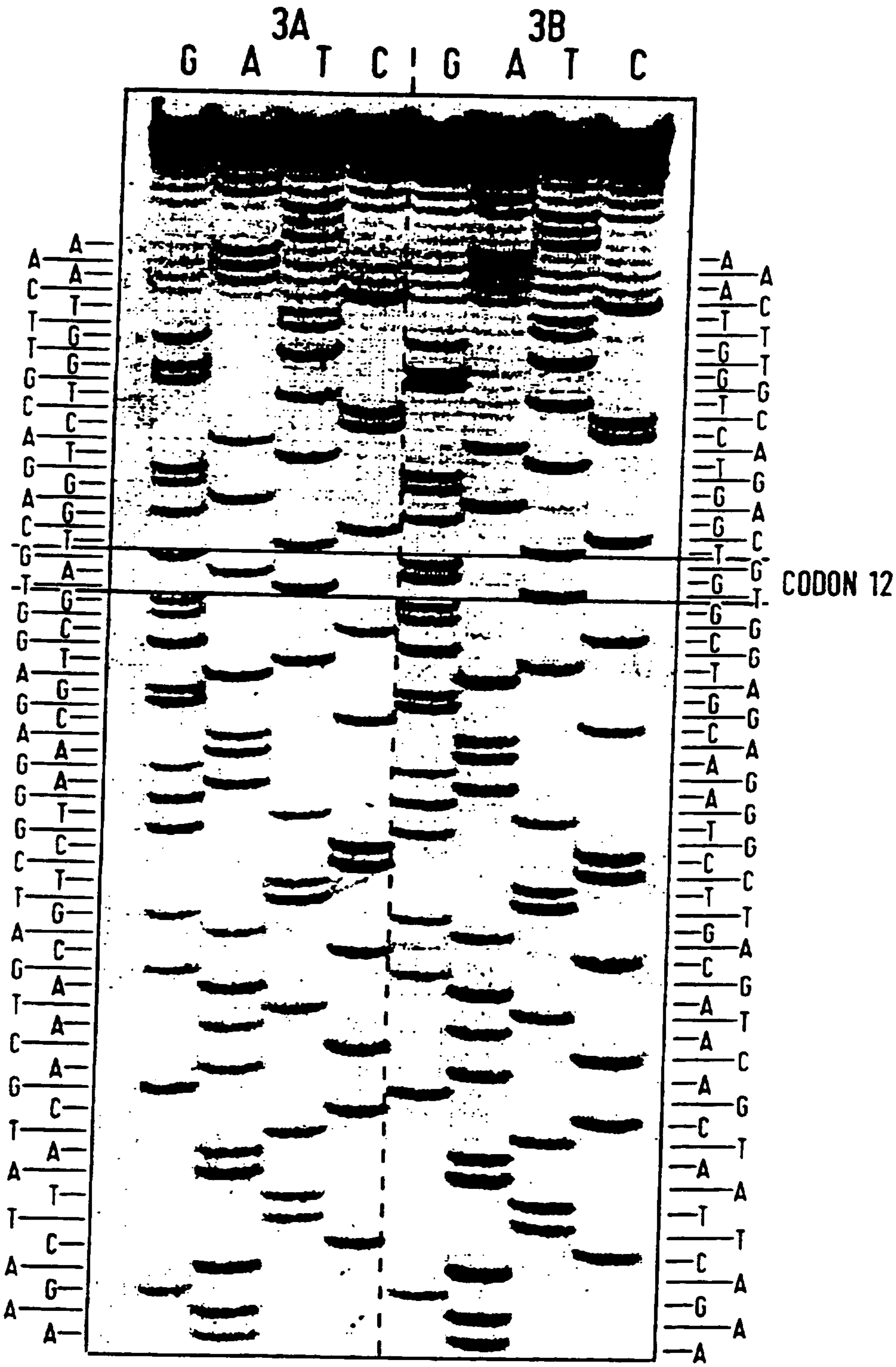


FIG. 5

FIG. 1

CCGCGGTCCGGTCCCGCTCCGGGTCAGAATTGGCGGCTGCGGGGACAGCCTTGCGGCTAGGCAGGGGCGGGGCCGCCGCGTGGG
TCCGGCAGTCCCTCCTCCCGCCAAGGCGCCGCCCAGACCCGCTCTCCAGCCGGCCCGGCTCGCCACCCTAGACCCCCCCAGCC
ACCCCTTCCTCCGCCGGCCCGGCCCCCGCTCCTCCCCCGCCGGCCCGGCCCGGCCCCCTCCTTCTCCCCGCCGGCGCTCGCTGC
CTCCCCCCTCTTCCCTCTTCCCACACCGCCCTCAGCCGCTCCCTCTCGTACGCCCGTCTGAAGAAGAATCGAGCGCGGAACGCA
TCGATAGCTCTGCCCTCTGCGGCCGCCCGGCCCCGAACTCATCGGTGTGCTCGGAGCTCGATTTTCCTAGGCGGCGGCCGCGGC
GGCGGAGGCAGCAGCGGCGGCGGCAGTGGCGGCGGCGAAGGTGGCGGCGGCTCGGCCAGTACTCCCGGCCCCCGCCATTTCGG
ACTGGGAGCGAGCGCGGCGCAGGCACTGAAGGCGGCGGCGGGGCCAGAGGCTCAGCGGCTCCCAGGTGCGGGAGAGAGGTACGG
AGCGGACCACCCCTCCTGGGCCCCTGCCCGGGTCCCGACCCTCTTTGCCGGCGCCGGGCGGGGCCGGCGGCGAGTGAATGAAT
TAGGGGTCCCCGGAGGGGCGGGTGGGGGCGCGGGCGCGGGGTCGGGGCGGCTGGGTGAGAGGGGTCTGCAG>>>GTACTGGT
GGAGTATTTGATAGTGTATTAACCTTATGTGTGACATGTTCTAATATAGTCACATTTTCATTATTTTTATTATAAG GCCTGC

												12	13							
	Met	Thr	Glu	Tyr	Lys	Leu	Val	Val	Val	Gly	Ala	<u>Gly</u>	<u>Gly</u>	Val	Gly	Lys	Ser	Ala	Leu	Thr
TGAAA	ATG	ACT	GAA	TAT	AAA	CTT	GTG	GTA	GTT	GGA	GCT	<u>GGT</u>	<u>GGC</u>	GTA	GGC	AAG	AGT	GCC	TTG	ACG

Ile	Gln	Leu	Ile	Gln	Asn	His	Phe	Val	Asp	Glu	Tyr	Asp	Pro	Thr	Ile	Glu
ATA	CAG	CTA	ATT	CAG	AAT	CAT	TTT	GTG	GAC	GAA	TAT	GAT	CCA	ACA	ATA	GAG *GTAAATCTTGTTT*

TAATATGCATATTACTGGTGCAGGACCATTCTTTGATACAGATAAAGGTTTCTCTGACCATTTTCATGAGT>>>ATCACCATTT
TACATTCCCACCAGCAATGCACAAAGATTTCAGTGTCTGTATCCTTGCTAACACTTATTTCCATTTTTTGAGTTTTTTTGTT
TTGTTTTTTTAATAATAGCCAATCCTAATGGGTATGTGGTAGCATCTCATGCTTTTGATTTTATTTTCCTGACTATTGATGATG
TTGAGCATCTTTTCAGGTGCTTAGTGGCCATTTGTCCGTCATCTTTGGAGCAGGAACAATGTCTTTTCAAGTCCTTTGCCCAT
TTTTAAATTGAATTTTTTTGTTGTTGAGTTGTATATAACACCTTTTTTGAAGTAAAAGGTGCACTGTAATAATCCAGACTGTGTT

	Asp	Ser	Tyr	Arg	Lys	Gln	Val	Val	Ile	Asp	Gly	Glu	Thr	Cys	Leu	Leu	Asp
TCTCCCTTCTCAG	GAT	TCC	TAC	AGG	AAG	CAA	GTA	GTA	ATT	GAT	GGA	GAA	ACC	TGT	CTC	TTG	GAT

						61											
Ile	Leu	Asp	Thr	Ala	Gly	<u>Gln</u>	Glu	Glu	Tyr	Ser	Ala	Met	Arg	Asp	Gln	Tyr	Met	Arg	Thr	Gly
ATT	CTC	GAC	ACA	GCA	GGT	<u>CAA</u>	GAG	GAG	TAC	AGT	GCA	ATG	AGG	GAC	CAG	TAC	ATG	AGG	ACT	GGG

Glu	Gly	Phe	Leu	Cys	Val	Phe	Ala	Ile	Asn	Asn	Thr	Lys	Ser	Phe	Glu	Asp	Ile	His	His	Tyr
GAG	GGC	TTT	CTT	TGT	GTA	TTT	GCC	ATA	AAT	AAT	ACT	AAA	TCA	TTT	GAA	GAT	ATT	CAC	CAT	TAT

Ar
AG *GTGGGTTTAAATTGAATATAATAAGCTGACATTAAGGAGTAATTATAGTTTTTATTTTTTGAGTCTTTGCTAATGCCATGC*
ATATAATATTTAATAAAAATTTTTAAATAATGTTTATGAGGTAGGTAATATCCCTGTTTTATAAATGAAGTTCTTGGGGGATT
AGAGCAGTGGAGTAACTTGCTCCAGACTGCATCGGTAGTGGTGGTGCTGGGATTGAAACCTAGGCCTGTTTGACTCCACAGCCT
TCTGT>>>TCTAGAATTTTTCAGTAGTTTCTGTTTTACTATTATGATCTACCTGCATATTAACCTATTAGGTTATAGTTTTAC
TATACTTCTAGGTATTTGATCTTTTGAGAGAGATACAAGGTTTCTGTTTAAAAAGGTAAAGAAACAAAATAACTAGTAGAAGAA
GGAAGGAAAATTTGGTGTAGTGGAAACTAGGAATTACATTGTTTTCTTTCAGCCAAATTTTATGACAAAAGTTGTGGACAGGT

q Glu Gln Ile Lys Arg Val
TTTGAAAGATATTTGTGTTACTAATGACTGTGCTATAACTTTTTTTTCTTTCCCAG A GAA CAA ATT AAA AGA GTT

Lys Asp Ser Glu Asp Val Pro Met Val Leu Val Gly Asn Lys Cys Asp Leu Pro Ser Arg Thr
AAG GAC TCT GAA GAT GTA CCT ATG GTC CTA GTA GGA AAT AAA TGT GAT TTG CCT TCT AGA ACA

Val Asp Thr Lys Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Phe Ile Gly Thr Ser
GTA GAC ACA AAA CAG GCT CAG GAC TTA GCA AGA AGT TAT GGA ATT CCT TTT ATT GAA ACA TCA

Ala Lys Thr Arg Gln
GCA AAG ACA AGA CAG GTAAGTAACACTGAAATAAATACAGATCTGTTTTCTGCAAAATCATAACTGTTATGTCATTTA
ATATATCAGTTTTTCTCTCAATTATGCTATACTAGGAAATAAAACAATATTTAGTAAATGTTTTTGTCTCTTGAGAGGGCATTG
CTTCTTAATC>>ACAGAAGACCCAGTCTCAGCTTCACTTGTATACCCTGGAAATAGACTGAAAGGTGTTAAAATTTAAAATAA
AACTCAAGGTTCCAGTTTCTTGACTCACCTTTGAGATTCTTTTATGTTTTTGTTTGTTTTTTAACAAAGGTTTCACGTCCATATT
TTACCATTTTTCTTCTCATTCTCCCCTGGAGGAGGGTGTGGGAATCGATAGTATATAAATCACTTTTTTTCCTAAGTCAAAGAA
GTAATTTAAAGCTAACTTCAGTTTAGGCTTTAATTCCAGGACTAGCAAACTAAAATGGTTGCATTAATTGACAAACAGATGCTA
ATACCTGTGTTTAGGCTTGTCATAATCTCTGCTAATTCCTAATTTAAAAATTTTAAAATTTAATTCCATTAGAAAACAAAACT
GACTTTTAAGAACAAACCAGGATTCTAGCCCATATTTAAAACTGCATCCTCAGTTTTATTCAAACAGTCTGATGTCTGTTTAA
AAAAAAAAAAATCTCAAGCTCATAATCTCAAACTTCTTGCACATGGCTTTCCCAGTAAATTACTCTTACCAATGCAACAGACT

Arg Val Glu Asp Ala Phe Tyr Thr Leu Val Arg Glu Ile Arg
TTAAAGAAGTTGTGTTTTACAATGCAG AGA GTG GAG GAT GCT TTT TAT ACA TTG GTG AGA GAG ATC CGA

Gln Tyr Arg Leu Lys Lys Ile Ser Lys Glu Glu Lys Thr Pro Gly Cys Val Lys Ile Lys Lys
CAA TAC AGA TTG AAA AAA ATC AGC AAA GAA GAA AAG ACT CCT GGC TGT GTG AAA ATT AAA AAA

Cys Ile Ile Met OC
TGC ATT ATA ATG TAA TGTG GTAAGTTTAAGTTCAGCACATTAATTTTGGCAGAAAGCAGATGTCTTTTAAAGGTAACA
AGGTGGCAACCACTTTAGAACTACTTAGGTGTAGTATTCTAACTTGAAGTATTAAAAGATAAGAAACTTGTTTCCATAATTAG
T>>>GAATTCTAAAAGTCCTAATATATGTAATATATATTCAGTTGCCTGAAGAGAAACATAAAGAATCCTTTCTTAATATTTTT
Gly
TCCATTAATGAAATTTGTTACCTGTACACATGAAGCCATCGTATATATTCACATTTTAATACTTTTTATGTATTTCAG GGT

Val Asp Asp Ala Phe Tyr Thr Leu Val Arg Glu Ile Arg Lys His Lys Glu Lys Met Ser Lys
GTT GAT GAT GCC TTC TAT ACA TTA GTT CGA GAA ATT CGA AAA CAT AAA GAA AAG ATG AGC AAA

Asp Gly Lys Lys Lys Lys Lys Lys Ser Lys Thr Lys Cys Val Ile Met OC
GAT GGT AAA AAG AAG AAA AAG AAG TCA AAG ACA AAG TGT GTA ATT ATG TAA ATACAATTTGTACTT

TTTTCTTAAGGCATACTAGTACAAG TGGTAATTTTTGTACATTACACTAAATTATTAGCATTTGTTTTAGCATTACCTAATTT
TTTTCCTGCTCCATGCAGACTGTTAGCTTTTACCTTAAATGCTTATTTTAAAATGACAGTGGAAGTTTTTTTTTCCTCGAAGT
GCCAGTATTCCCAGATTTTTGGTTTTTGAACTAGCAATGCCTGTGAAAAAGAAACTGAATACCTAAGATTTCTGTCTTGGGGTT
TTTGGTGCATGCAGTTGATTACTTCTTATTTTTCTTACCAAGTGTGAATGTTGGTGTGAAACAAATTAATGAAGCTT

FIG. 1 CONT'D

FIG. 2

```
                    Xcm I
                    CCA---------TGG

                    BstX I
                    CCA------TGG

           5K1
[------------------CCA------>

TGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTG>>>
ACTTTTACTGACTTATATTTGAACACCATCAACCTCGACCACCGCATCCGTTCTCACGGAACTGCTATGTCGATTAAGTCTTAGTAAAAC<<<

>>>TGGACGAATATGATCCAACAATAGAGGTAAATCTTGTTTTAATATGCATATTACTGGTGCAGGACCATTCTTTGATACAGATAAAGGTTT
<<<ACCTGCTTATACTAGGTTGTTATCTCCATTTAGAACAAAATTATACGTATAATGACCACGTCCTGGTAAGAAACTATGTCTATTTCCAAA

                                                            3K1
                                       <---------------------------]

                                   3K2
                        <-----C-------------C---------]

                              Xcm I
                              CCA---------TGG

                              BstX I
                              CCA------TGG

                  3K3
              <---------------]
```

Primers:

5K1: ACTGAATATAAACTTGTGGTCCATGGAGCT

5K1-Bio: Biotin-ACTGAATATAAACTTGTGGTCCATGGAGCT

3K1: TTATCTGTATCAAAGAATGGTCCTGCACCA

3K2: GAATGGTCCTCCACCAGTAATATGGATATTA

3K3: TATTAAAACAAGATTTAC

Endonucleases:

```
        C C A N N N N N|N T G G
BstX I: G G T N|N N N N N A C C

       C C A N N N N N|N N N N T G G
Xcm I: G G T N N N N|N N N N N A C C
```

FIG. 3

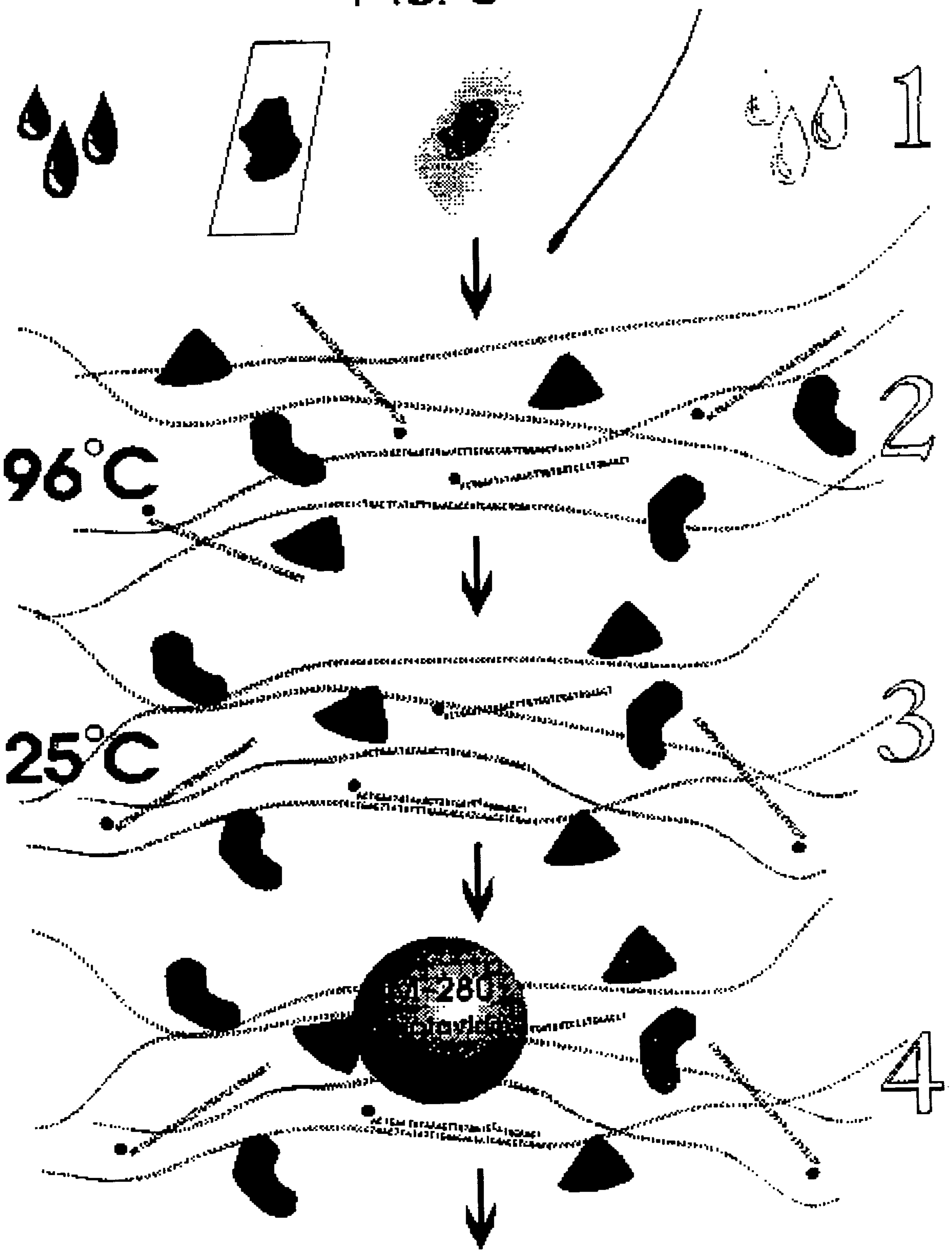


FIG. 3 CONT'D

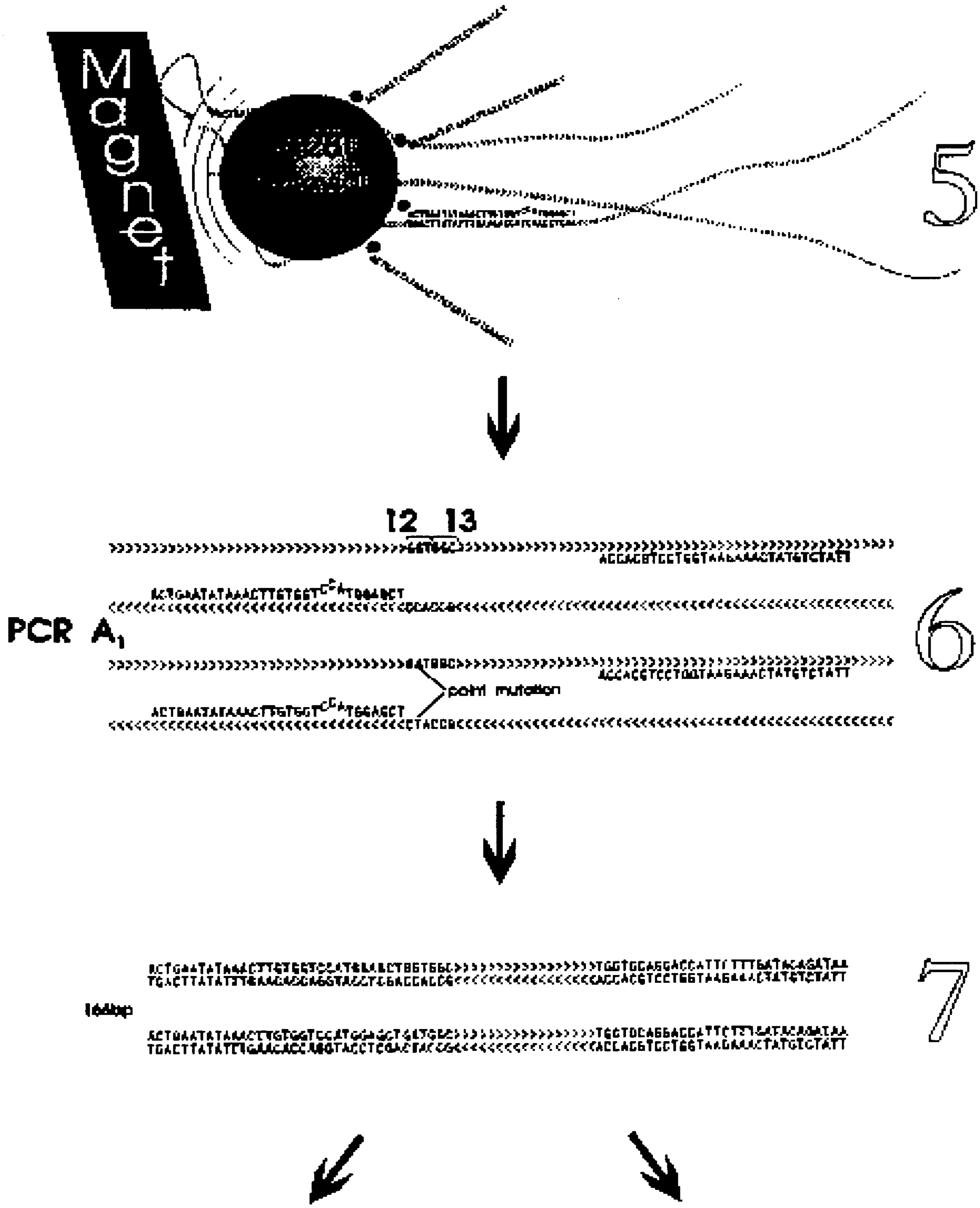


FIG. 3 CONT'D

6 / 10

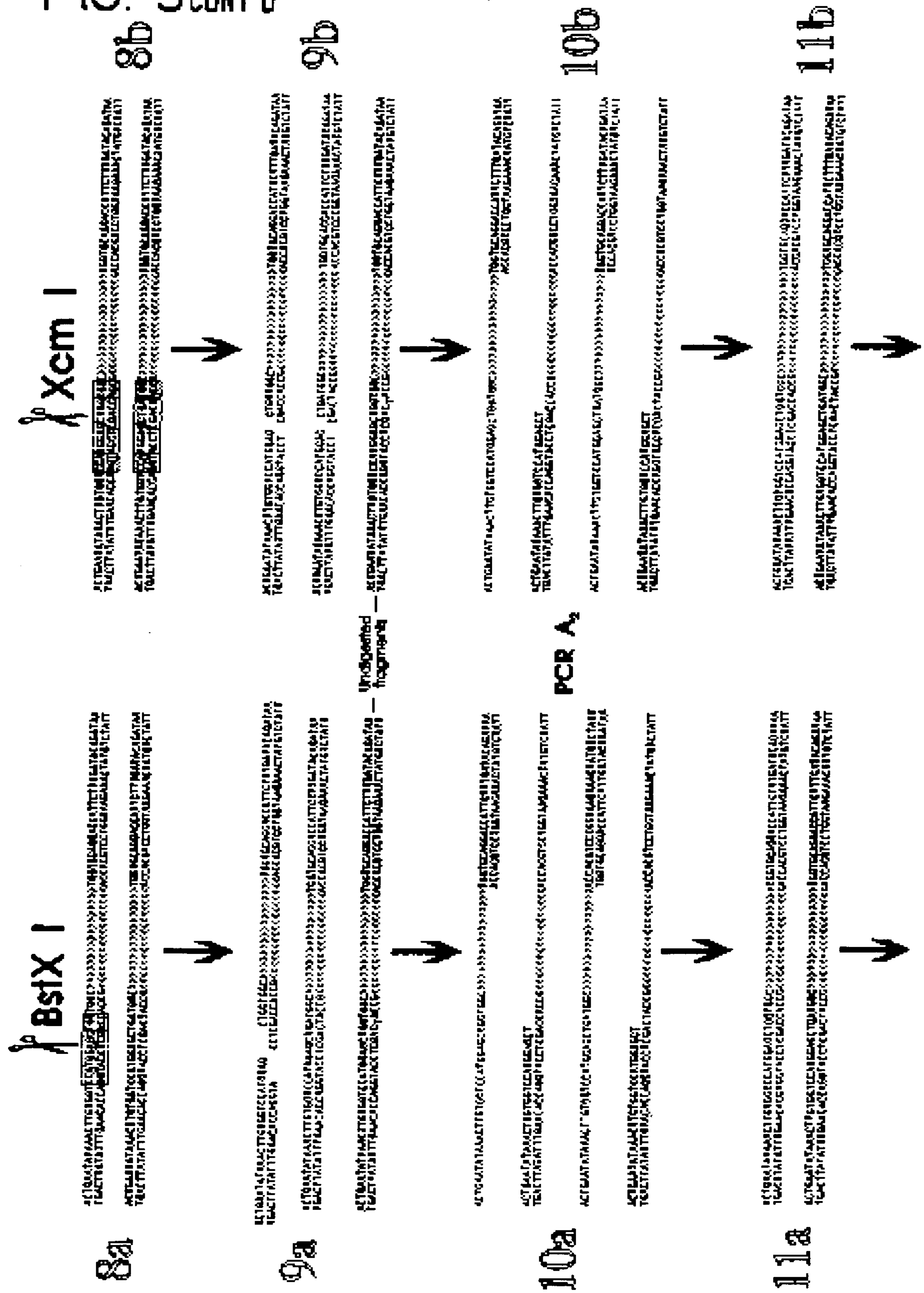
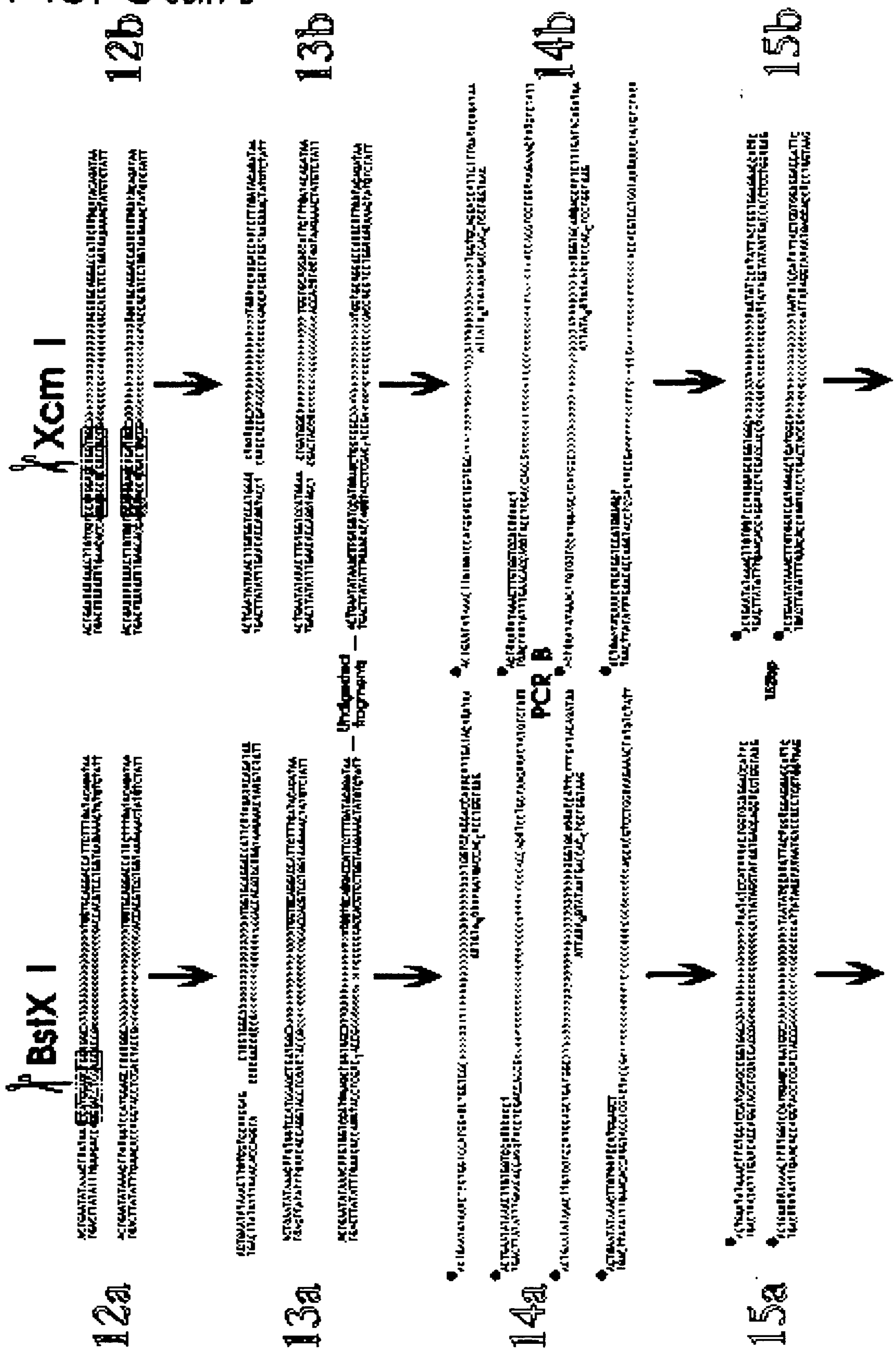
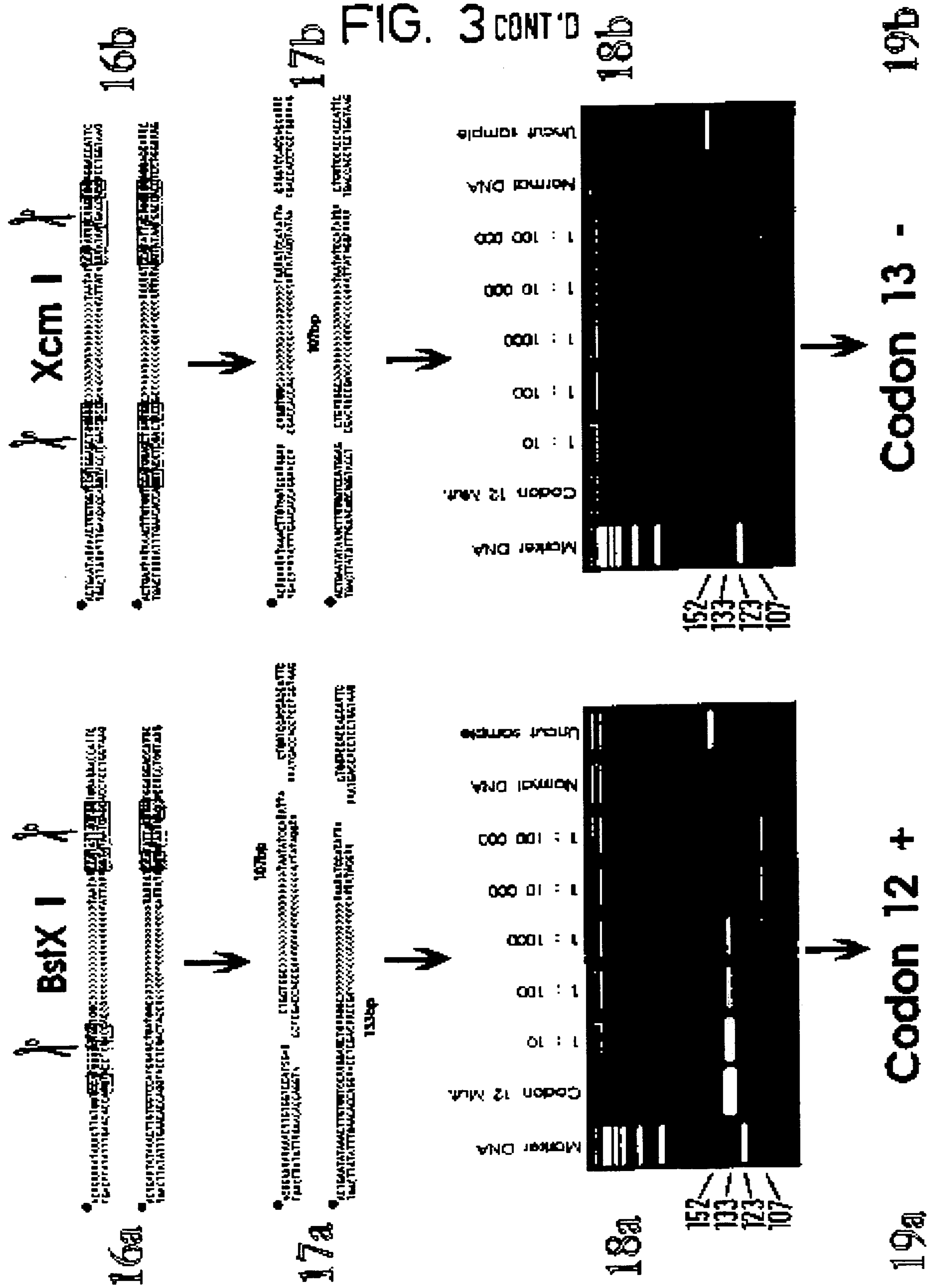


FIG. 3 CONT'D


12a
12b
BslX I
Xcm I
13a
13b
Unligated fragments
14a
14b
PCR B
15a
15b
150bp

8/10

FIG. 3 CONT'D



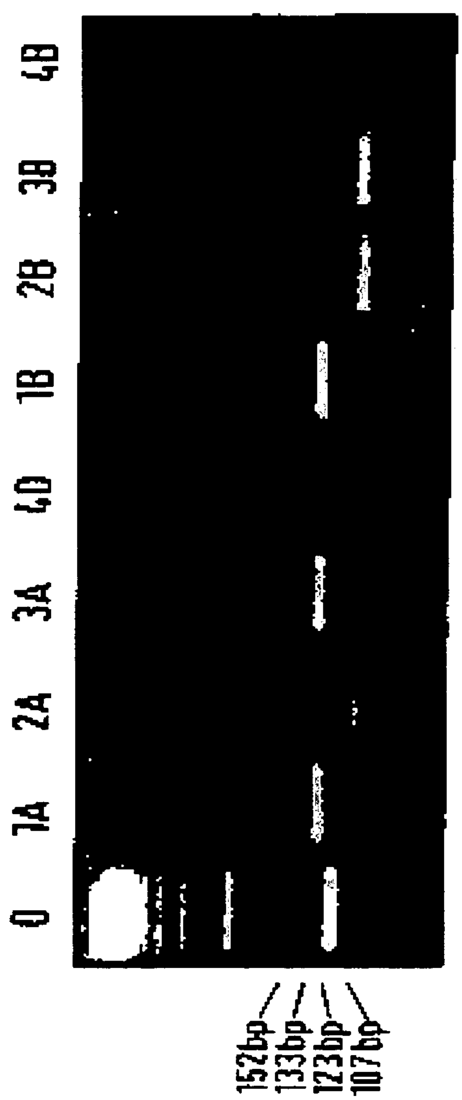


FIG. 4

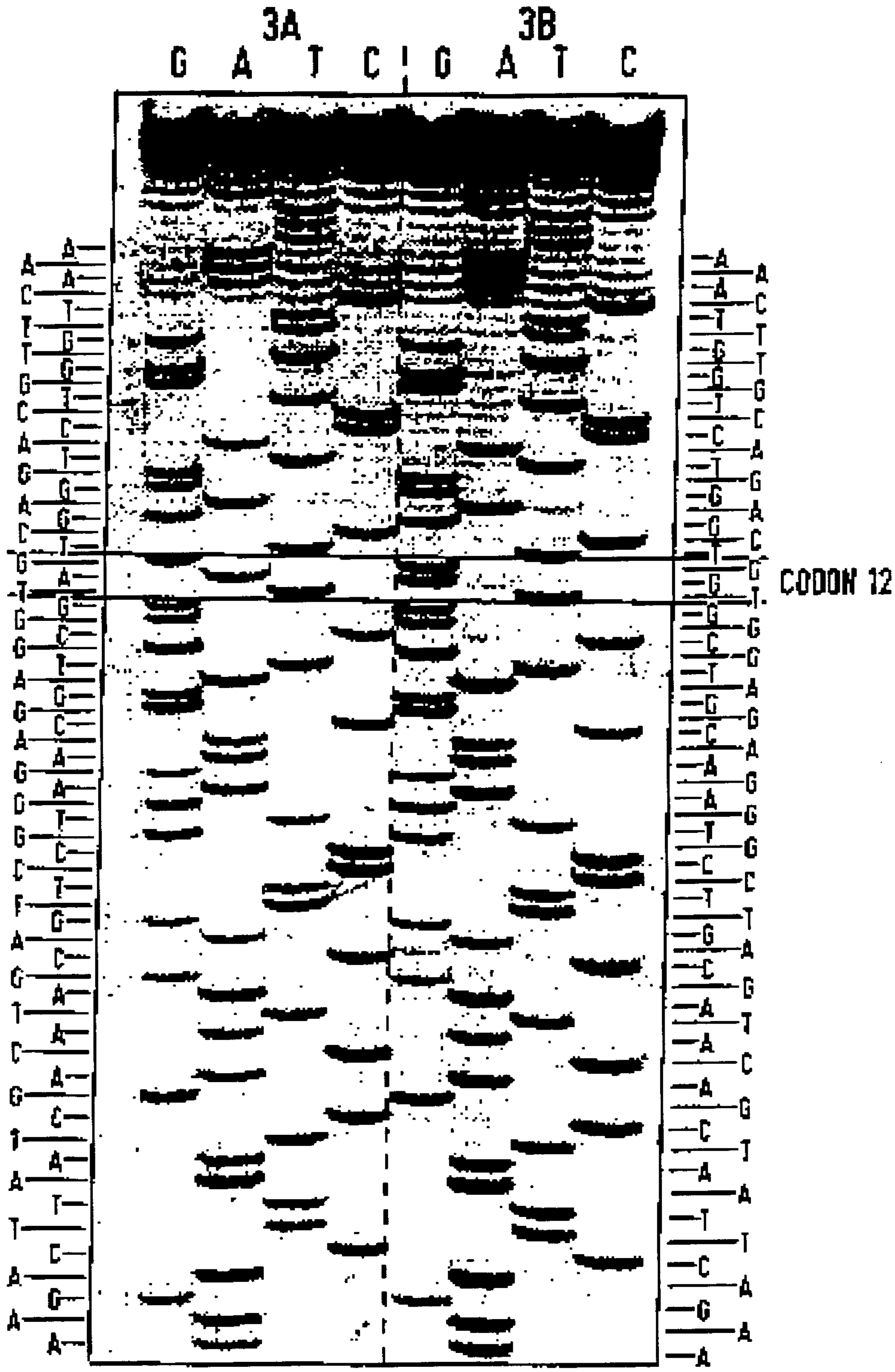


FIG. 5

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, C07H 21/04, C12P 19/34 C12P 1/44

A3

(11) International Publication Number: WO 96/15262

(43) International Publication Date: 23 May 1996 (23.05.96)

(21) International Application Number: PCT/GB95/02644

(22) International Filing Date: 10 November 1995 (10.11.95)

(30) Priority Data:
9422814.5 11 November 1994 (11.11.94) GB

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(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published
With international search report.

(88) Date of publication of the international search report: 15 August 1996 (15.08.96)

(54) Title: METHOD FOR THE DETECTION OF *RAS* ONCOGENES, IN PARTICULAR THE 12-*RAS* ONCOGENE

(57) Abstract

The invention relates to an oligonucleotide primer sequence for use in *in vitro* amplification, characterised in that said primer sequence is capable of creating a *BstX* I restriction site overlapping codon (12) and/or an *Xcm* I restriction site overlapping codon (13) or a *Bce* 831 restriction site overlapping codon (61) of the wild-type K-*ras* oncogene, methods of using said primer sequences for detecting activating mutations in codons (12 and/or 13 and/or 61) of the K-*ras* oncogene and kits for performing the methods.

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INTERNATIONAL SEARCH REPORT

In[illegible]tional Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04 C12P19/34 C12Q1/44

According to International Patent Classification (IPC) or to both national classification and IPC

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AM. J. CLIN. PATHOL, vol. 100, no. 6, December 1993, pages 686-89, XP000572107 LIN S ET AL: "mutation analysis of K-ras oncogenes in gasteroenterologic cancers by the amplified created restriction sites method" see the whole document ---	1-22
A	ONCOGENE, vol. 6, no. 8, 1991, pages 1353-62, XP002004427 MITSUDOMI T ET AL: "Mutations of ras genes distinguish a subset of non-small-cell lung cancer lines from small-cell cancer cell lines" see the whole document ---	1-22
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Date of the actual completion of the international search	Date of mailing of the international search report
31 May 1996	07. 06. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Osborne, H

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PCR METHODS AND APPLICATIONS, vol. 1, no. 2, 1991, pages 146-48, XP000572412 JACOBSON D ET AL: "Rapid nonradioactive screening for activating ras oncogene mutations using PCR-primer introduced restriction analysis (PCR-PIRA)" see the whole document ---	1-22
A	EP,A,0 466 083 (E.R. SQUIB AND SONS INC.) 15 January 1992 see the whole document -----	1-22

INTERNATIONAL SEARCH REPORT

Informa[illegible] patent family members

I[illegible]tional Application No
PC[illegible] 95/02644

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP-A- 4293485	19-10-92